VOLUME 55 [J. CELL. AND COMP. PHYSIOL.] — NUMBER 2

UNIVERSITY OF ILLINOIS
LIBRARY
OCT 7 1960

JOURNAL OF
CELLULAR AND COMPARATIVE
PHYSIOLOGY

Board of Editors

Arthur K. Parpart, Managing Editor
Princeton University

W. R. Amberson
University of Maryland

H. F. Blum
National Cancer Institute

D. W. Bronk
The Rockefeller Institute

L. B. Flexner
University of Pennsylvania

M. H. Jacobs
University of Pennsylvania

D. Marsland
New York University

D. Mazia
University of California

APRIL 1960

PUBLISHED BIMONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
Thirty-sixth Street at Spruce, Philadelphia 4, Pa.

Second class postage paid at Philadelphia, Pa.

Publications of The Wistar Institute

THE JOURNAL OF MORPHOLOGY

Devoted to the publication of original research on animal morphology, including cytology, protozoology, and the embryology of vertebrates and invertebrates. Articles do not usually exceed 32 journal pages in length.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE JOURNAL OF COMPARATIVE NEUROLOGY

Publishes the results of original investigations on the comparative anatomy and physiology of the nervous system.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE AMERICAN JOURNAL OF ANATOMY

Publishes the results of comprehensive investigations in vertebrate anatomy — descriptive, analytical, experimental.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE ANATOMICAL RECORD

Organ of the American Association of Anatomists and the American Society of Zoologists

For the prompt publication of concise original articles on vertebrate anatomy, preliminary reports; technical notes; critical notes of interest to anatomists and short reviews of noteworthy publications.

Issued monthly, 3 vols. annually: $30.00 Domestic, $32.00 Foreign, per year.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

Publishes papers embodying the results of original researches of an experimental or analytical nature in the field of zoology.

Issued 9 times a year, 3 vols. annually: $30.00 Domestic, $32.00 Foreign, per year.

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

Publishes original articles on comparative human morphology and physiology as well as on the history of this branch of science and the techniques used therein. In addition, it gives comprehensive reviews of books and papers, a bibliography of current publications, abstracts and proceedings of the American Association of Physical Anthropologists, and informal communications.

Issued quarterly, 1 vol. annually: $10.00 Domestic, $11.00 Foreign, per year.

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

Publishes papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE JOURNAL OF NUTRITION

Official Organ of the American Institute of Nutrition

Publishes original research bearing on the nutrition of any organism. Supplements to the regular monthly issues are published irregularly as required by the editorial board.

Issued monthly, 3 vols. annually: $22.50 Domestic, $24.00 Foreign, per year.

THE AMERICAN ANATOMICAL MEMOIRS

Publishes original monographs based on experimental or descriptive investigations in the field of anatomy which are too extensive to appear in the current periodicals. Each number contains only one monograph. List of monographs already published, with prices, sent on application.

These publications enjoy the largest circulation of any similar journals published.

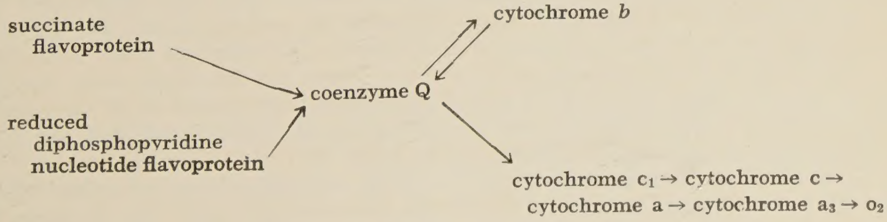
Send Subscriptions and Business Correspondence to

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
THIRTY-SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

Cytochromes of Muscles of Marine Invertebrates

A. L. TAPPEL
Department of Food Science and Technology, University of California, Davis, California

Generalizations on the cytochrome pattern in the terminal electron transport chain of mitochondria are based primarily on spectral (Chance and Williams, '56) and chemical isolation (Green, '59) studies of heart and liver mitochondria of higher mammals. These studies lead to the formulation of an electron transport chain of the type shown below:


succinate flavoprotein
reduced diphosphopyridine nucleotide flavoprotein
coenzyme Q
cytochrome b
cytochrome c₁ → cytochrome c →
cytochrome a → cytochrome a₃ → o₂

Since the concentrations of cytochromes a_3, a, b, c and c_1 are similar and a chain of this type is compatible with kinetic data, it is the simplest formulation. However, chemical studies suggest that the arrangement of the electron transport chain may be more complex. Spectral studies of the cytochromes in amphibian cardiac muscle (Ramirez, '59) show that the relative concentrations of cytochromes a_3, a, b and $c + c_1$ in intact muscle are similar to those of isolated mitochondria of the amphibian and higher mammals.

There have been few quantitative studies of the cytochromes of the electron transport chain in invertebrates; those on the fly being important exceptions. Sarcosomes of the flight muscle of the fly have the cytochromes a_3, a, b and c occurring in about the same proportion as in higher mammals (Chance and Sactor, '58). A literature survey (Gumbmann et al., '58) of the cytochromes and terminal electron transport in marine animals shows that existing information is very meager. In studies of the pathways of terminal respiration in marine invertebrates, Ghiretti-Magaldi et al., ('57, '58) and Ghiretti et al., ('59) used difference spectra of particulate preparations for qualitative identification of cytochromes a_3, a, b and c. Furthermore, cytochrome a_3 was shown to be the terminal oxidase by carbon monoxide inhibition and spectral studies, and cytochrome c of *Aplysia* was purified and found to be spectrally the same as mammalian cytochrome c. Because many of the marine invertebrates have muscles containing little or no intermuscular pigments, like the myoglobin of mammals which interferes with direct spectral measurement of the intact cytochromes, they offer great advantages for study.

This paper reports comparative spectrophotometric studies of the cytochromes of some crustaceans and bivalve mollusks. They are defined qualitatively by difference and low temperature spectra and measured quantitatively from difference spectra.

MATERIALS AND METHODS

The crustaceans used in this study were: blue crab, *Callinectes sapidus;* American lobster, *Homarus americanus;* and shrimp, *Pandalus* species. The bivalve mollusks were: eastern oyster, *Gassostrea virginica;* northern quahog clam, *Mercenaria mercenaria;* and bay and sea scallop, *Pectin irradians* and *Pectin magellanicus*. These invertebrates were collected through commercial fisheries. All were alive at the time samples were taken, except shrimp which were obtained frozen and scallop muscles which were obtained refrigerated and frozen. The crabs, lobsters, oysters and clams were sacrificed just prior to the measurement of difference spectra. After the crabs and lobster muscles and hearts were dissected out they were carefully washed with lobster Ringer solution. Muscles of crab and lobster were selected on the basis of apparent cytochrome content and size. For shrimp, the muscles were selected on the basis of availability and apparent cytochrome content. From the crab flexor muscle, the tan-colored muscles of highest cytochrome content were selected, dissected out, and cut into 1-mm slices. The tan-colored muscles highest in cytochrome content were dissected from the coxopodite of the 4 ambulatory legs of the lobster. They were either cut into 1-mm slices or homogenized and suspended in 50% glycerol. The 50% glycerol suspension of muscle homogenate proved useful because its high viscosity held the ground muscle in suspension and it also made the muscle preparation translucent. Muscle preparations in 50% glycerol were required for the measurement of low temperature spectra. Muscles from the basipodite and endopodite of the pleopods of shrimp were pressed out and ground in a homogenizer in 50% glycerol. Oyster adductor muscles were cut into 1-mm slices and some were homogenized and suspended in 50% glycerol. Various other preparations of oyster were made, especially from the mantle and gills. Clam adductor and foot muscles were cut into 1-mm slices. Scallop adductor muscles were pooled and then homogenized in 50% glycerol.

The various muscles and muscle slices were mounted and gently compressed between two perforated lucite plates as described by Ramirez ('59). This mounting left an uncovered cylinder of stretch muscle 5.5 mm diameter. The thickness of the muscle or muscle slice, between 0.3 and 1 mm, was not completely uniform. The differences in light transmission were corrected by adjusting the amount of light reaching the phototube of the spectrophotometer. For difference spectra, identical samples of muscle were mounted on the lucite plates which were contained in a 1 cm cuvette holding appropriate Ringer's solution. The two samples were first oxygenated with pure oxygen gas and a base line determined. Then one muscle sample was reduced using various reductants and the difference spectra of the muscle were obtained with a split-beam spectrophotometer, as described by Chance ('54). Low temperature spectra of muscle homogenate in 50% glycerol were recorded using the apparatus and techniques described by Estabrook ('56). A temperature of −190°C was maintained with liquid nitrogen. The monochrometer was calibrated with cytochrome *c*. Half band widths were about 0.5 mμ. Because of the low cytochrome content of some of the muscles of marine invertebrates, plexiglass cuvettes with 3 mm optical paths were used as well as 1-mm cuvettes. This method has previously been used for measuring the low temperature spectra of the cytochromes of mitochondria (Estabrook and Mackler, '57); no difficulty was encountered using muscles homogenized in 50% glycerol.

RESULTS

The cytochromes contained in these marine invertebrate muscles are qualitatively determined by difference spectra and low temperature spectra. The recording of low temperature spectra is the most selective method known for identification of cytochromes. There is peak sharpening and splitting at the low temperature and intensification, especially of the α peaks, due to multiple reflection of the samples in the microcrystalline state. Difference spectra which are typical of the best obtained are given in figures. The peaks in the difference spectra for many of the samples examined are tabulated in tables 1 and 2. Cytochromes are identified by the position of the α and γ peaks in the room temperature difference spectra and the α, β and γ peaks in the low temperature spectra using the spectral characteristics established for mammalian liver and heart mitochondria where the cytochromes *a*, a_3, *b*, *c* and c_1 have been most fully studied (Estabrook and Mackler, '57; Chance and Williams, '55; Chance and Baltscheffsky, '58). From the best difference spectra, approximate concentration of the respiratory pigments are calculated and are recorded in table 3.

The concentrations are calculated using the wave-length pairs and changes in mo-

TABLE 1

Wavelength (mμ) position of peaks in difference spectra

Preparation	Reductant	α			β	Flavo-protein	γ			
		Cyt. *a*	Cyt. *b*	Cyt. $c + c_1$	Cyt. $c + b$	min.	Cyt. a_3	Cyt. *c*	Cyt. a_3CO max.	Cyt. a_3CO min.
Crab flexor muscle 1	Anaerobiosis	600	560sh.	549	513	469	443	417		
Crab flexor muscle 2	Dithionite	603	560sh.	549	519	465	442			
	Anaerobiosis	602	560sh.	549	519	467				
Crab flexor muscle 3	Anaerobiosis	604	562sh.	549		470	443	418	426	445
Crab heart muscle 1	Anaerobiosis	604	560sh.	550	520	465	444	418		
Crab heart muscle 2	Anaerobiosis	603	560sh.	548	518	465	444	421		
Lobster ambulatory leg muscle 1	Anaerobiosis	602	562sh.	548	517	490	441	418sh.		
Lobster ambulatory leg muscle 2	Succinate	601	560sh.	548	518	463	439	420sh.	426	445
Lobster muscle 3 in in 50% glycerol	Dithionite	603	563sh.	550	519	465	443		426	443
Lobster heart muscle	Anaerobiosis	604	563sh.	550	520	465	443			
	Succinate	602	561sh.	550	518	465	441	419sh.	425	444
Shrimp pleopod muscle in 50% glycerol	Dithionite	602	558	550	520	465	442		427	444
Shrimp pleopod muscle in 50% glycerol	Dithionite	602	560	550	520	465	443			

TABLE 2

Wavelength (mμ) position of peaks in difference spectra

Preparation	Reductant	α			β	Flavo-protein min.	γ				
		Cyt. *a*	Cyt. *b*	Cyt. $c + c_1$	Cyt. $c + b$		Cyt. a_3	Cyt. *b*	Cyt. *c*	Cyt. a_3CO	
										max.	min.
Scallop adductor muscle	Dithionite		–557–			463	445sh.	–427–			
Scallop adductor muscle in 50% glycerol	Succinate			548	519	460	445sh.	–428–			
Scallop adductor muscle in 50% glycerol	Dithionite		558	550sh.	527sh.	455		–425–			
Scallop adductor muscle in 50% glycerol	Dithionite	605	560sh.	549	520			–427–		412	433
Scallop adductor muscle centrifuged fraction	Dithionite	605		549	517			–425–			
Oyster adductor muscle	Dithionite			550				–425–			
Oyster adductor muscle in 50% glycerol	Succinate		557sh.	549	524sh.	445			423		
	Dithionite		–555–		530sh.	458			423	415	439
Oyster homogenate	Anaerobiosis			548		457			420		
Oyster organs, particulate preparation	Dithionite			552		465		–430–			
Oyster mantle and gills, homogenate	Dithionite			550					422		
Oyster mantle and gills, particulate preparation	Dithionite			549		459		–432–			
Clam adductor muscle 1	Anaerobiosis			555	520	454			418		
Clam adductor muscle 2	Succinate	600		552				–435–			
Clam adductor muscle 3	Succinate		–562–						420	418	437
Clam foot muscle	Succinate			550		465		–430–			

TABLE 3

Concentrations of the respiratory pigments of marine invertebrates

Muscle	Sample size	Respiratory pigments μ moles/kg wet weight				
		Cyt. *a*	Cyt. a_3	Cyt. *b*	Cyt. $c + c_1$	Flavoprotein
Crab flexor	2 crabs	7.5	8.7	1.5	4.8	19.0
Lobster ambulatory leg	3 lobsters	8.0	8.8	0.7	5.1	11.6
Scallop adductor	2 pools of 21 gm each	(0.3)	(0.3)	1.2	1.8	5.5
Oyster adductor	pool of 10 oysters	0.08	0.5	1.0	1.9	5.3
Clam adductor	4 clams	(0.08)	(0.8)	5.3	3.7	3.8

lecular extinction coefficients determined by Chance and Williams ('56). These are as follows: cytochrome *a*, 605–630 mμ, 16 $cm^{-1} \times mM^{-1}$; cytochrome *b*, 564–575 mμ, 20 $cm^{-1} \times mM^{-1}$; cytochrome *c*, 550–540 mμ, 19 $cm^{-1} \times mM^{-1}$; flavoprotein, 465–510 mμ, 11 $cm^{-1} \times mM^{-1}$; and cytochrome a_3, 445–465 mμ, 91 $cm^{-1} \times mM^{-1}$ for the respiratory pigment, the pair of wavelengths used to measure absorbancy changes, and the changes in molecular extinction coefficients, respectively. Present knowledge of the cytochromes *a* is insufficient to resolve the problem of whether there is one cytochrome, a_3 the terminal oxidase, or two cytochromes, *a* and a_3. Spectral studies by Chance and Yonetani ('59) indicate two cytochromes, *a* and a_3, but isolation studies by Ambe and Venkataraman ('59) indicate a single cytochrome of the *a* type. For consistency with previous spectral studies the cytochromes *a* will be treated assuming cytochromes *a* and a_3 with the reservation that these two measurements may, in reality, be of one cytochrome.

For comparative purposes the calculated concentration of flavoprotein is given in table 3. However, in this study specific qualitative tests for flavoproteins were not made. The occurrence of a minimum at 465 mμ in the difference spectra (tables 1 and 2) is characteristic of flavoprotein. Riboflavin is found widely distributed in marine animals (Gumbmann et al., '58). In addition, riboflavin and flavine adenine dinucleotide were found (Ghiretti-Magaldi et al., '58) in all the tissues of *Octopus vulgaris* so there are no reasons to doubt the presence of flavoproteins in the marine invertebrates examined here. The results tabulated in tables 1, 2 and 3, as well as those in the form of difference and low temperature spectra, will be discussed for each invertebrate.

Crab

A typical room temperature difference spectrum of crab flexor muscle is shown in figure 1. Qualitatively, this spectrum is very similar to difference spectra of mammalian mitochondria and amphibian muscles. The large 604 mμ peak corresponds to the α peak of cytochromes $a + a_3$. The 560 mμ shoulder corresponds to the α peak of cytochrome b. The sharp 550 mμ peak is the α peak of cytochromes $c + c_1$. The β peak of cytochromes $c + b$ is at 519 mμ. The trough at 465 mμ is characteristic of flavoproteins. The γ peak at 443 mμ corresponds to that of cytochrome a_3. Because of the large concentration of cytochromes $a + a_3$, their γ peaks mask the γ peaks of cytochromes *b* and *c*. The difference spectrum of crab heart in figure 2 shows that it is qualitatively similar to flexor muscle. Unlike flexor muscle it does not have the high concentration of cytochromes $a + a_3$ compared to cytochromes *b* and *c*. Difference spectra for crab flexor muscle and heart muscle were obtained on a number of samples under a variety of reducing conditions. The resolution in all cases was very good, and the peaks in the difference spectra are tabulated in table 1. The close correspondence of the peaks in the difference spectra for various samples and methods of reduction offers good checks on this qualitative identification. For the sample treated with carbon monoxide, the 426 mμ maximum and the 445 mμ minimum are characteristic of the carbon monoxide derivative of cytochrome a_3.

Although room temperature difference spectra of crab muscle show sufficient definition for a reasonable qualitative identi-

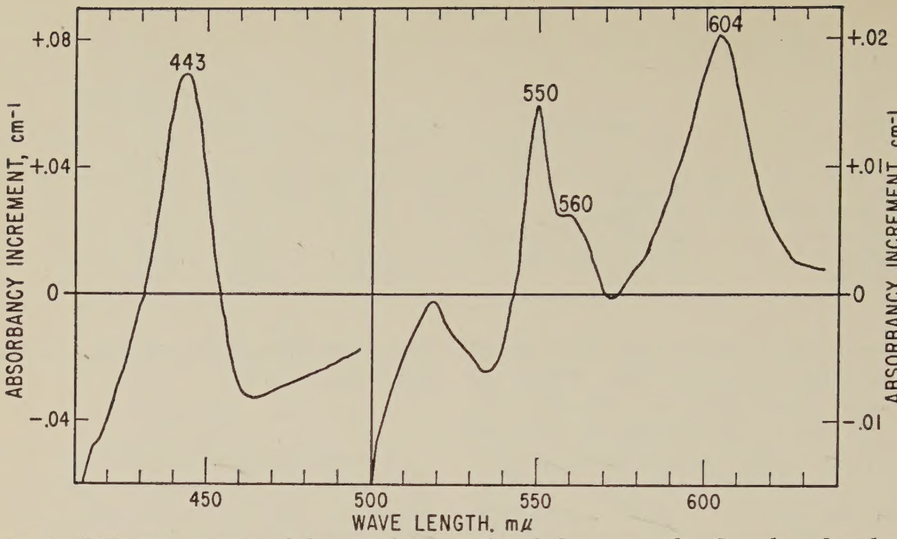


Fig. 1 Difference spectra of the cytochromes of crab flexor muscle. Sample reduced with succinate and N_2 gas, reference oxidized with O_2 gas, muscle slice 1 mm thick.

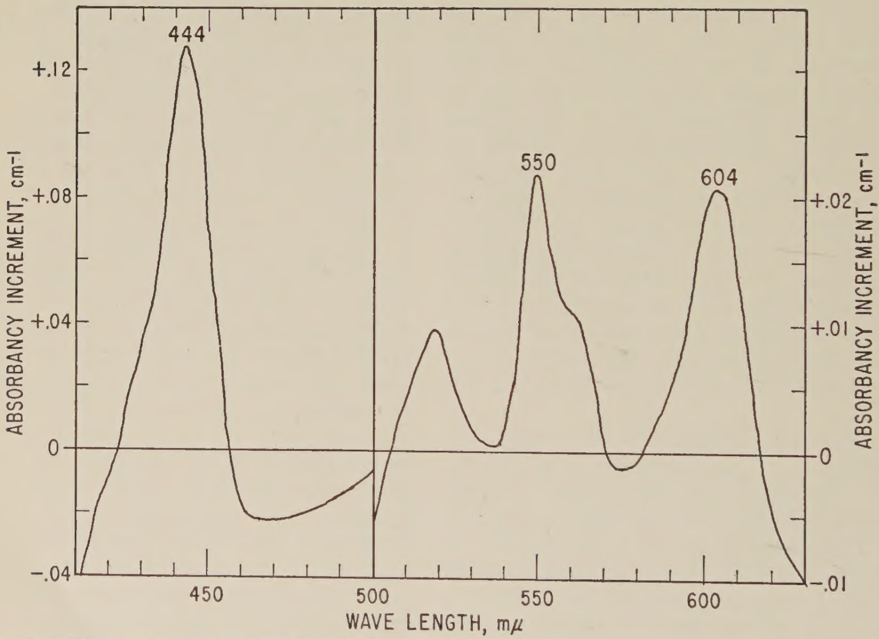


Fig. 2 Difference spectra of the cytochromes of crab cardiac muscle. Sample was anaerobic, N_2 gas; reference was aerobic, O_2 gas. Muscle was 0.7 mm thick.

fication, the best qualitative identification comes from low temperature spectra. The low temperature spectrum in figure 3 shows very good definition for a direct determination on a muscle. The sharp peak at 602 mμ is identified as the α peak of cytochromes $a + a_3$. The sharp peak at 548.8 mμ is identified as that of cytochrome c, and is in the same position as that of heart muscle cytochrome c at 548.6 mμ. The smaller peaks are identified as those of cytochrome c_1 at 556 mμ and cyto-

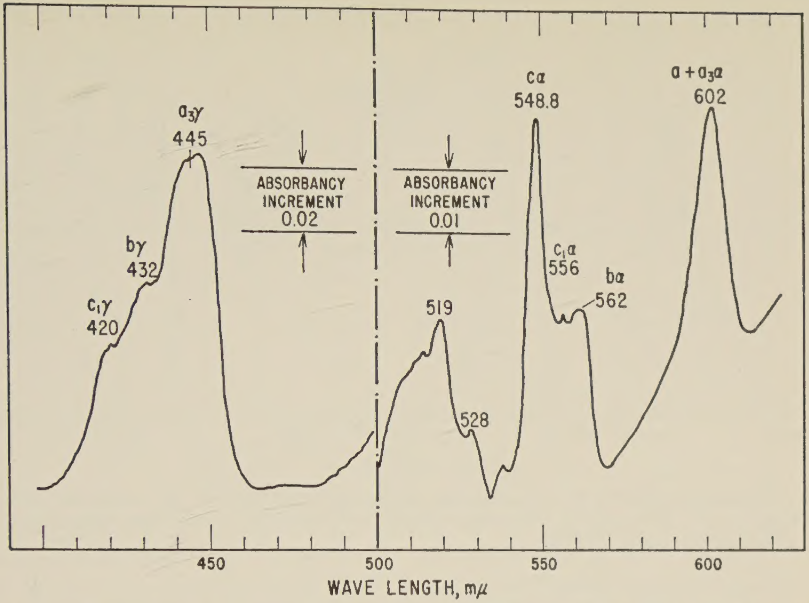


Fig. 3 Low temperature spectra of cytochrome components of crab muscle. Six-tenths gram of crab muscle in 4 ml 50% glycerol, optical path 1 mm, sample reduced with dithionite, reference in oxidized state.

chrome *b* at 562 mμ. The β peaks at 519 mμ and 528 mμ correspond to those of cytochrome *c* and *b*, respectively. Unusual definition is found in the γ peaks where 445 mμ corresponds to cytochrome a_3, 432 mμ to cytochrome *b*, and 420 mμ to cytochrome c_1. Thus, this low temperature spectrum gives good qualitative evidence for the presence of cytochromes $a + a_3$, *b*, *c* and c_1 in crab muscle.

The quantitative amounts of the respiratory pigments in crab muscles were calculated from two good difference spectra like that shown in figure 1. As can be seen from the data in table 3 and the height of the peaks in figure 1, crab muscle contains a large amount of cytochromes $a + a_3$ and a relatively small amount of cytochrome *b*.

Lobster

The data obtained by measuring the difference spectra of various lobster muscle preparations, using a variety of reductants, are shown in figures 4 and 5 and table 1. By the same procedure used in qualitatively identifying the cytochromes of crab muscle, these difference spectra were compared to difference spectra for the known cytochrome components of mammalian liver and heart mitochondria. These data allow the identification of cytochromes $a + a_3$, *b*, and *c*. There is close correspondence between the peaks maxima in the difference spectra, table 1. Carbon monoxide treatment of the lobster ambulatory leg and cardiac muscle gave good spectral evidence of the carbon monoxide derivative of cytochrome a_3 with a maximum at 426 mμ and a minimum at 443–445 mμ.

The low temperature spectra of lobster muscle (fig. 6) shows good definition allowing the qualitative identification of cytochromes $a + a_3$, *b* and *c*. Low temperature spectra for cytochromes reduced with either succinate and dithionite show the 600 mμ peak of cytochromes $a + a_3$ the 560 mμ peak of cytochrome *b* and the 548.6 mμ peak of cytochrome *c*. There is also resolution of the β peaks of cytochrome *c* at 519 mμ and of cytochrome *b* at 528 mμ. No spectral evidence was found for the occurrence of cytochrome c_1 in lobster. This cytochrome is either absent or present in such low quantity that its α peak is obscured by the α peaks of cytochromes *c* and *b*. The con-

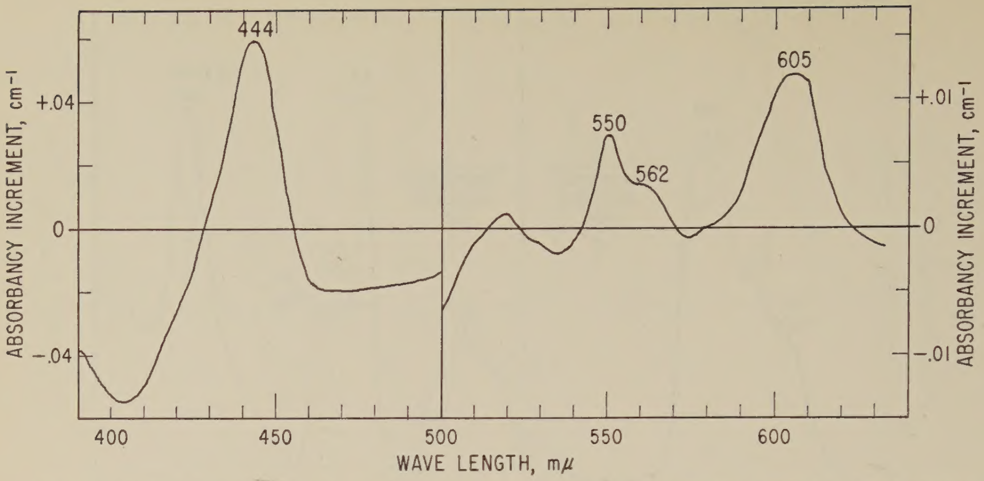


Fig. 4 Difference spectra of cytochromes of lobster ambulatory leg muscles. Five grams of lobster muscle in 20 ml 50% glycerol, sample reduced with succinate, reference oxidized with O_2 gas, optical path 10 mm.

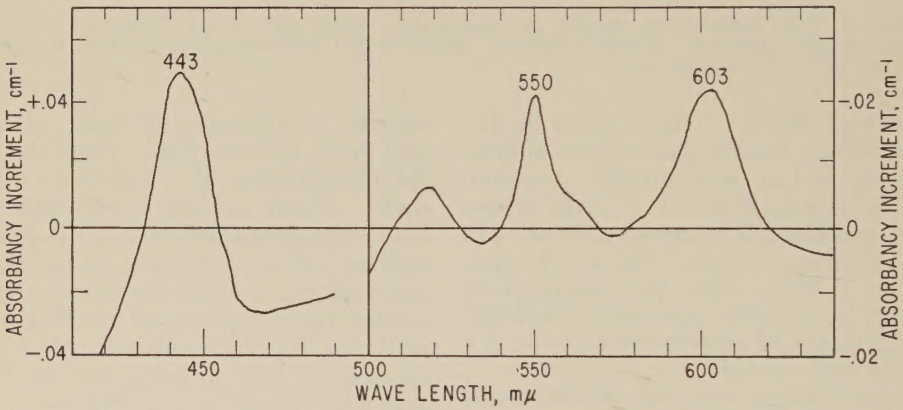


Fig. 5 Difference spectra of the cytochromes of lobster cardiac muscle. Sample was anaerobic, N_2 gas; reference was aerobic, O_2 gas. Muscle was 0.3 mm thick.

tent of cytochrome a_3 is so large that only its peak (445 mμ) is found.

From three difference spectra like that shown in figure 4 the concentration of the respiratory pigments in the ambulatory leg of lobster was calculated and the results are shown in table 3. As can be seen in the difference and low temperature spectra the similarities in the composition and concentration of respiratory pigments in lobster and crab muscles are striking. Lobster, like crab, has very high cytochromes $a + a_3$ relative to b and c. The cytochrome b content is only one tenth that of the cytochromes $a + a_3$. Comparison of the difference spectra in figures 4 and 5 shows that lobster leg muscle has a higher proportion of cytochromes $a + a_3$ (603–605 mμ) than does cardiac muscle.

Shrimp

The difference spectrum of the cytochromes of shrimp pelopod muscles in figure 7 indicates the occurrence of cytochromes $a + a_3$, as shown by the height of the 603 mμ peak, and the relatively high concentration of cytochrome b indicated by the 560 mμ peak which is equal to the 550 mμ peak of cytochrome c. These characteristics were consistent in all of the

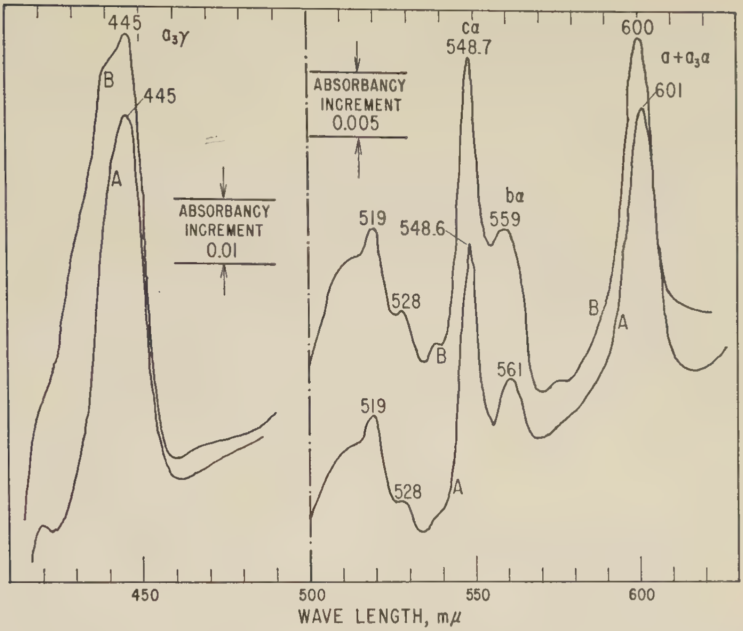


Fig. 6 Low temperature spectra of cytochrome components of lobster muscle. Curve A, reduced with succinate. Curve B, reduced with dithionite. Two grams lobster muscle in 12 ml 50% glycerol, optical path 1 mm, reference cuvette contained lobster muscle in oxidized state.

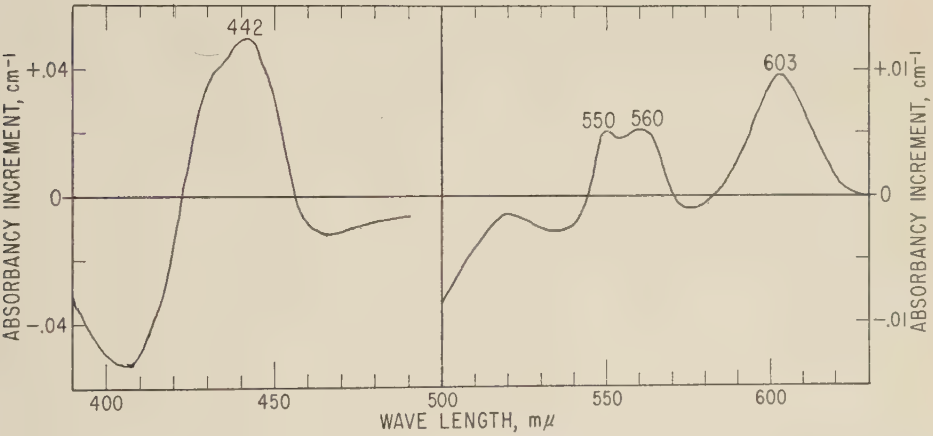


Fig. 7 Difference spectra of cytochromes of shrimp pleopod muscles. About 0.1 gm of shrimp muscle in 5 ml 50% glycerol, sample reduced with dithionite, reference oxidized with O_2 gas, optical path 10 mm.

samples of pooled muscle which were studied (table 1). The spectral characteristics of the carbon monoxide treated sample (table 1) are evidence for the occurrence of cytochrome a_3.

The low temperature spectra (fig. 8) of shrimp muscle show good resolution in the α region. A high content of cytochromes $a + a_3$ is indicated by the high peak at 599 mμ. Cytochrome *c* is identified by its characteristic peak at 548.5 mμ and cytochrome c_1 by its characteristic peak at 554 mμ. Judging by the α peaks at 558 mμ and 563 mμ there are two cytochromes

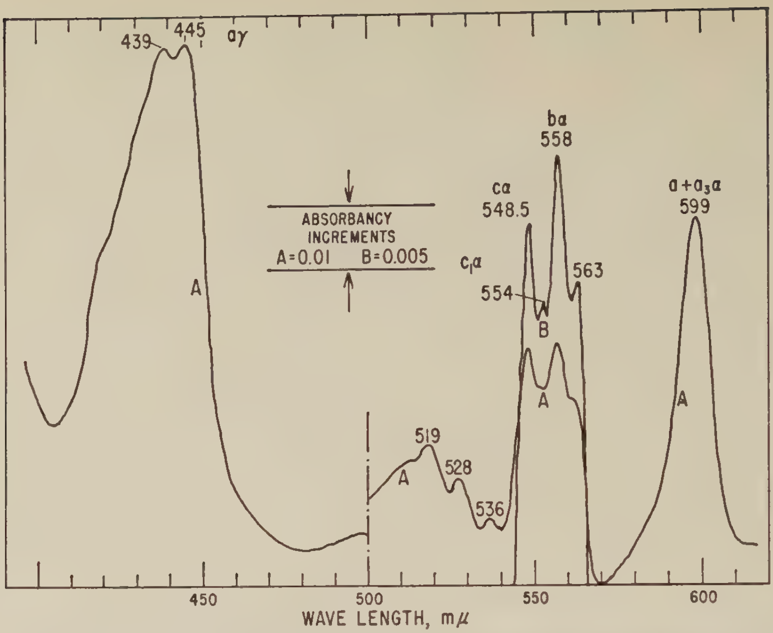


Fig. 8 Low temperature spectra of cytochrome components of shrimp muscle. Optical path 1 mm, sample reduced with dithionite, reference in oxidized state.

of the *b* type. The one which is spectrally most similar to the cytochrome *b* of mammals is that which has a large peak at 558 mμ and a β peak at 528 mμ. Presumably, it is a different cytochrome of the *b* type that has an α absorption peak at 563 mμ, a β peak at 536 mμ and a γ peak at 439 mμ. From these data one cannot identify this cytochrome, and this problem remains to be resolved until purification and further characterization can be achieved. The concentrations of these respiratory pigments in the muscles of shrimp were not computed because the sample size was not large enough for reliable estimates. The difference spectra of shrimp show that the cytochromes $a + a_3$ are present in larger amounts than the cytochromes *c*, c_1 and *b*.

Scallops

A typical difference spectrum of the cytochromes of scallop adductor muscle is shown in figure 9. It is apparent from this difference spectrum and the characteristics of the other difference spectra of scallop muscle (table 2) that this muscle contains cytochromes *a*, *b* and *c*. The high concentration of *c* usually obscures the α peak of cytochrome *b* so that it shows up as a slight shoulder in the α region. In the γ region the absorbance peak is mainly that of *b;* the small amount of *a* not being apparent. The quantitative results on respiratory pigments of scallops (table 3) show this high concentration of $c + c_1$ and *b* with the low calculated concentration of cytochromes $a + a_3$. The occurrence of cytochromes *c*, c_1 and *b* with spectral characteristics similar to that of the cytochromes in mammalian mitochondria was confirmed by low temperature spectra, as shown in figure 10. The α peaks of cytochrome *c* at 548.5 mμ, of cytochrome c_1 at 555 mμ and of cytochrome *b* at 560 mμ are clearly seen. Beta peaks for cytochromes *b* and *c* are shown at 529 and 518 mμ, respectively. The 433 mμ maximum in the γ region is probably that of cytochrome *b*.

The low temperature spectra of scallop muscle show some unusual characteristics. Firstly, the absorbance at 610 mμ must correlate with the difference spectrum absorbance of 606 mμ and this pigment would be classified as a cytochrome of the *a* type. That this may not be a cytochrome of the *a* type similar to that occurring in mammals is shown by the maximum 610 mμ

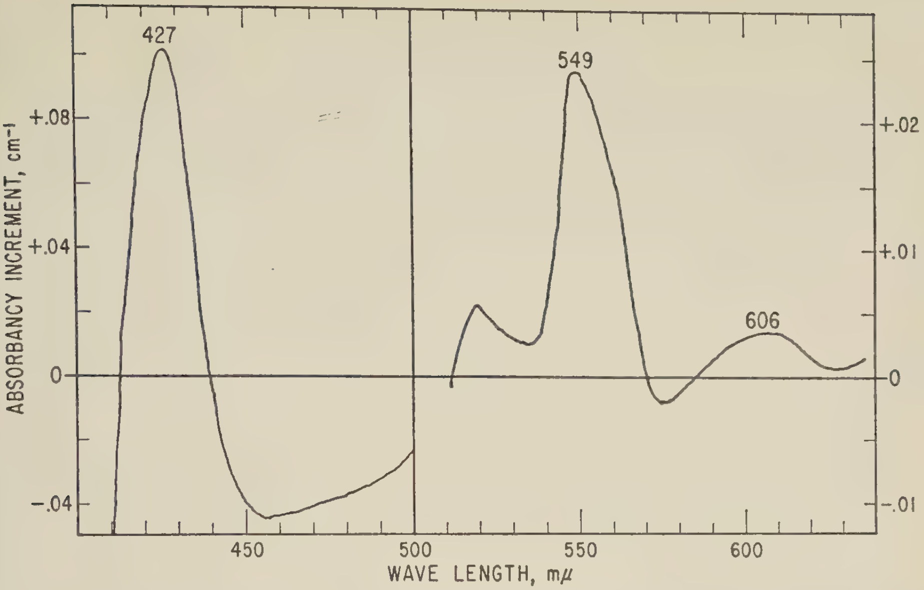


Fig. 9 Difference spectra of cytochromes of scallop adductor muscle. Twenty-three grams of scallop muscle in 50 ml 50% glycerol, sample reduced with dithionite, reference in oxidized state, optical path 10 mm.

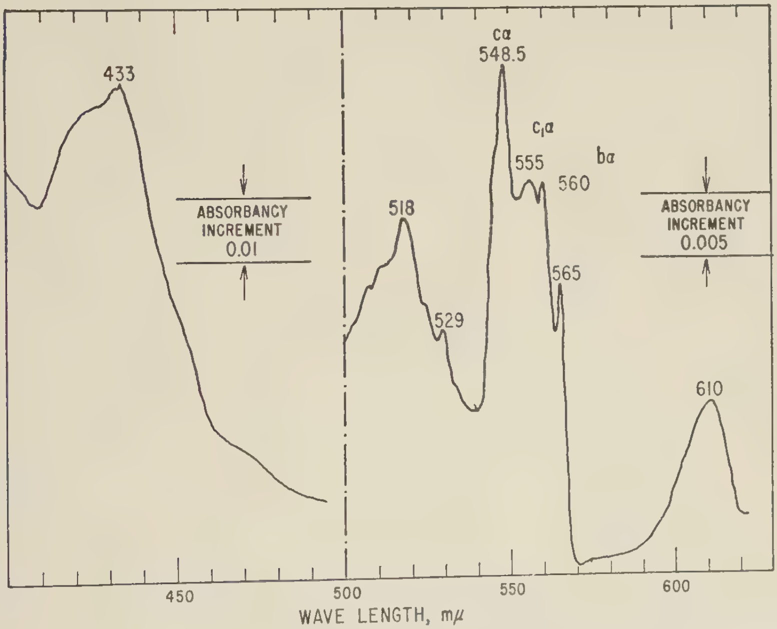


Fig. 10 Low temperature spectra of cytochrome components of scallop muscle. Seven grams of scallop muscle in 20 ml 50% glycerol, optical path 3 mm, sample reduced with dithionite, reference in oxidized state.

of the low temperature spectrum and the maximum and minimum peaks of the carbon monoxide compound (table 2) occurring at 412 mμ and 433 mμ. Secondly, another pigment which cannot be identified completely is that which gives rise to the absorbance at 565 mμ in the low temperature spectrum. Presumably this is another cytochrome of the *b* type besides the cytochrome *b* with maximum absorption at 560 mμ.

Oysters

Difference spectral studies of the cytochromes of oyster adductor muscle and other tissues (fig. 11 and table 2) show one α peak at 550 mμ to 556 mμ and one γ peak at 420 to 430 mμ. This indication of the occurrence of cytochromes *c* and *b* from difference spectra is reinforced by the more positive identification by low temperature spectrum (fig. 12). In the low temperature spectrum there is a major peak at 560 mμ of cytochrome *b*, and cytochrome *c* shows up as a minor peak at 548.6 mμ. Beta peaks for cytochrome *b* and *c* are also present at 526 and 518 mμ, respectively. As can be seen from the difference spectrum and the low temperature spectrums the concentrations of cytochromes $a + a_3$ is small. Also, the absorption characteristics of the carbon monoxide derivatives are different from those of the carbon monoxide derivative of the classical cytochrome a_3. Of the variety of oyster tissues examined, the adductor muscle was chosen as the best for measurement of the absorption spectra of cytochromes because it contains little other pigmentation. Also, it was most useful for comparison with scallop and clam adductor muscles. The concentration of respiratory pigments are shown in table 3; there is a low concentration of the cytochromes $a + a_3$ relative to *b* and *c*. There is considerable similarity between oyster and scallop adductor muscle.

Clams

Of the bivalve mollusks examined, the clams presented the most difficulty because of their bright-pink pigmentation. From the characteristics of the difference spectra shown in figure 13 and table 2 the occurrence of cytochromes *c* and *b* can be inferred from the maxima of the α and the γ peaks. Cytochrome *b* can be identified in the low temperature spectrum (fig. 14) with maximum at 562 mμ. There is no strong evidence for the occurrence of cytochromes $a + a_3$ since there is no maximum absorption in the 600 mμ region in the

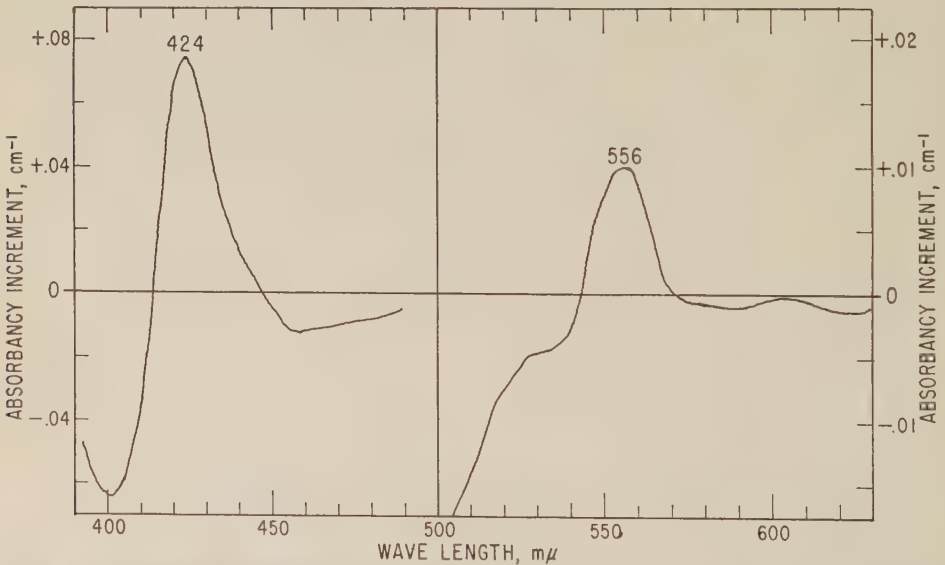


Fig. 11 Difference spectra of cytochromes of oyster adductor muscle. Nine grams oyster muscle ground in 28 ml 50% glycerol, sample reduced with dithionite, reference in oxidized state, optical path 10 mm.

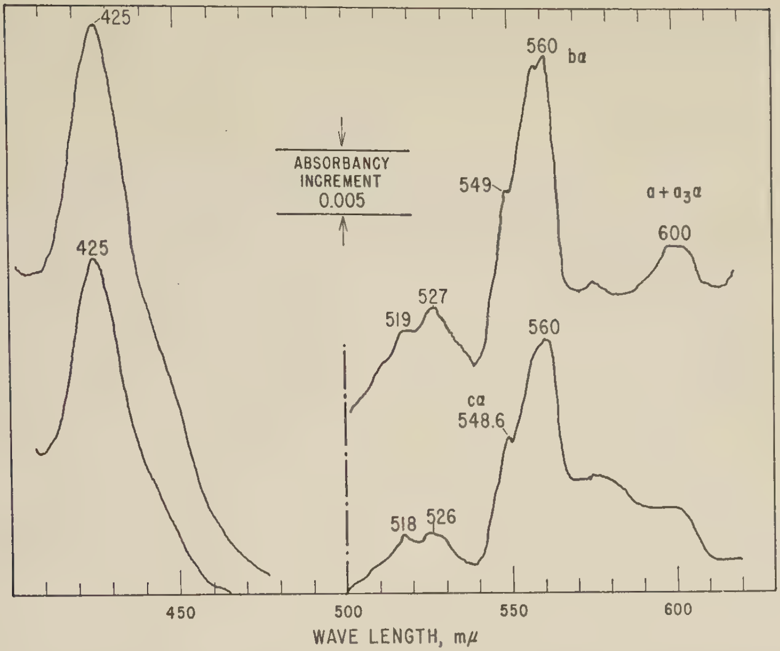


Fig. 12 Low temperature spectra of cytochrome components of oyster muscle. Nine grams of oyster muscle in 28 ml 50% glycerol, optical path 3 mm, samples reduced with dithionite, reference in oxidized state.

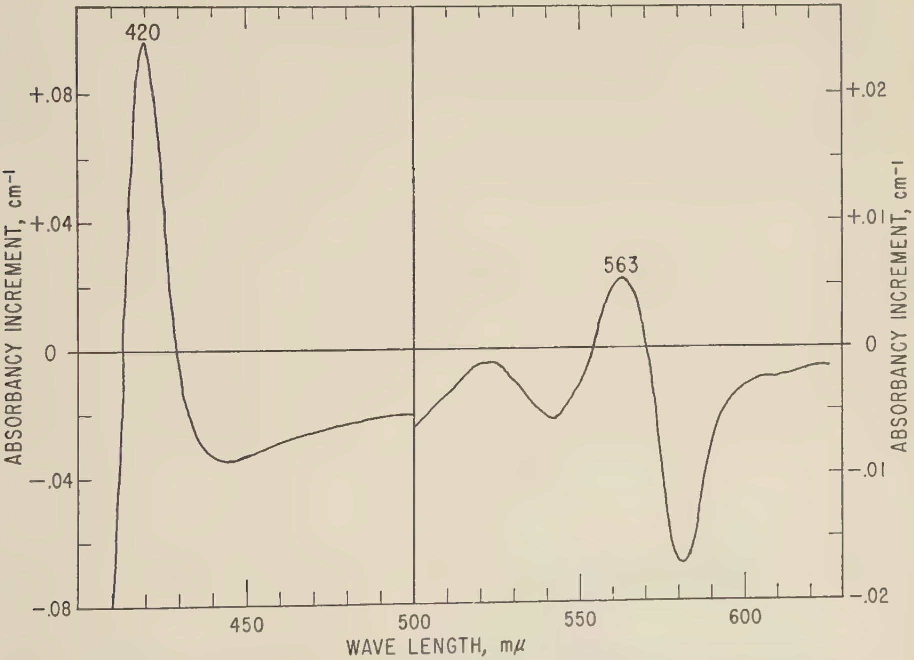


Fig. 13 Difference spectra of clam adductor muscle. Sample reduced with succinate and N_2 gas, reference oxidized with O_2 gas, muscle slice 1 mm thick.

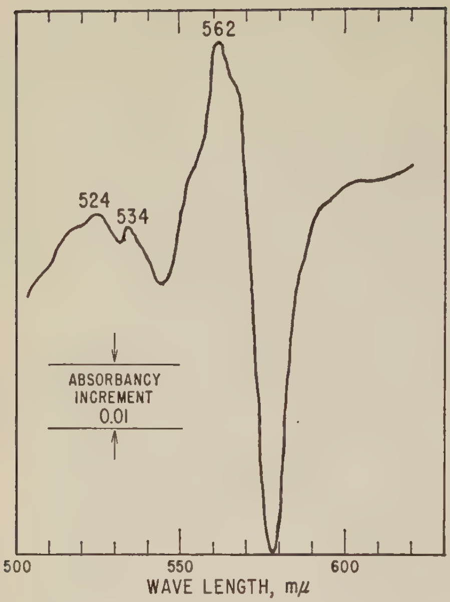


Fig. 14 Low temperature spectra of clam muscle. Three grams of clam muscle in 16 ml 50% glycerol, optical path 3 mm, sample reduced with dithionite, reference in oxidized state.

difference spectrum (fig. 13) or the low temperature spectrum (fig. 14). The positions of the carbon monoxide maximum and minimum are similar to those of oyster and scallop. The concentrations of the respiratory pigments show in table 3 that the cytochromes *b* and *c* are high while the calculated concentration of cytochromes $a + a_3$ is low.

DISCUSSION

Qualitative identification of the positions of the peaks in the low temperature spectra appeared to be the best method for identifying cytochromes $a_3 + a$, *b*, *c* and c_1 since the positions of absorbance maxima are available for these known cytochromes in mammalian mitochondria. From the large peaks and their positions shown in figures 3, 6 and 8, we can be reasonably certain that the three crustacea examined have the cytochromes $a + a_3$, *b*, *c* and c_1 with the exception that lobster has a very small amount or no c_1. Identification of cytochromes cannot be so clearly made in some of the bivalve mollusks. In the scallop (fig. 10) cytochromes *b*, *c* and c_1 can be identified. In the oyster (fig. 12) *b* is clearly seen, and there is spectral evidence for *c* and $a + a_3$. Heretofore, the occurrence of cytochrome *c* in oyster tissues has remained in doubt. Kawai ('58) found evidence of cytochrome *b* in oyster by low temperature visual spectroscopy. He also obtained cyanide and carbon monoxide inhibition evidece for the occurrence of cytochromes a_3. He was unable to find evidence for occurrence of cytochrome *c*.

The low temperature spectral method is a good technique for screening for cytochromes different from those which normally occur in mammalian mitochondria, but not for their identification, since there has been insufficient low temperature spectral studies on most of the other cytochromes. Thus, the low temperature spectrum of shrimp (fig. 8) has unassigned peaks at 563, 536 and 439 mμ probably corresponding to a cytochrome of the *b* type. The low temperature spectral study of the cytochromes of scallop muscle gave unassigned peaks at 610 and 565 mμ which appeared to correspond to a cytochrome of the *a* and of the *b* type, respectively. Further spectral and chemical characterization of these cytochromes is needed before they can adequately be identified. The cytochromes found here, which correspond to those normally found in muscle mitochondria, appear to be members of the electron transport systems in the mitochondria of these invertebrate muscles.

Evidence for this is our knowledge of the cytological locations of cytochromes, being in the mitochondria, of muscle cells and of kinetic studies on the cytochromes in intact muscles. The reduction by anaerobiosis and/or with succinate shows that the cytochromes respond as would be expected for members of electron transport systems in the mitochondria.

These are the most divergent cytochrome patterns found in animals. Whereas the mitochondria and muscles of higher mammals have cytochromes with ratios of their concentration near unity, these marine invertebrates have very different compositions. Lobster and crab have a low cytochrome *b* content compared to cytochromes $a + a_3$ and *c*. Scallop and oyster have a very low content of cyytochromes of the *a* type compared to cytochromes *b* and *c*. The relative concentration of cytochrome *b* is especially interesting since the function of cytochrome *b* is less understood than that of cytochromes $a + a_3$, *c* and c_1. Presently, cytochrome *b* is considered not to be on the main pathway of electron transport. The relatively large amount of cytochromes $a + a_3$ in lobster and crab act as a reservoir of oxidizing equivalents, and may be important in crustacea which have no other apparent reservoir of oxidizing equivalents in the muscle other than the oxygen brought in by the circulating hemocyanin of the blood.

The unusual composition of relatively high amounts of cytochromes *b* and *c* compared to cytocromes $a + a_3$ makes oysters, scallops and clams interesting for further studies; however, the relatively low total cytochrome content makes direct measurements difficult. On the basis of this study, scallops appear to be an invertebrate of choice for further study because of their relatively high content of cytochromes *b* and *c*, the presence of unidentified cytochromes of the *a* and *b* type, and the availability of large quantities of scallop muscle.

SUMMARY

The cytochromes of muscles of marine invertebrates have been studied by direct spectral measurements at liquid nitrogen and room temperatures. Qualitative and quantitative measurements allowed comparison of the cytochromes of invertebrate muscle mitochondria with the more intensively studied mammalian mitochondria. The marine invertebrates showed the most divergent cytochrome patterns thus far found in animals.

Low temperature spectra show the presence of cytochromes $a + a_3$, *b* and *c* in lobster, crab and shrimp. Cytochrome c_1 was found in crab and shrimp but not in lobster. Lobster and crab have a low cytochrome *b* content compared to cytochromes $a + a_3$ and *c*, and the content of cytochromes $a + a_3$ is relatively high compared to cytochrome *c*.

The bivalve mollusks, scallops, oysters and clams had low total concentrations of cytochromes with the concentrations of $a + a_3$ being very low compared to b and $c + c_1$. Improved resolution of the cytochromes of oyster by low temperature spectra showed the presence of cytochrome *c*, which could not be found in previous investigations.

ACKNOWLEDGMENTS

Part of this research was performed while the author was on sabbatical leave at the Johnson Foundation for Medical Physics, University of Pennsylvania. The author wishes to thank Prof. Britton Chance for use of the facilities of the Foundation and for his continued advice and assistance. The help and advice of Drs. J. Ramirez, J. Packer and P. Gonse is appreciated.

LITERATURE CITED

Ambe, K. S., and A. Venkataraman 1959 Depolymerization of cytochrome oxidase to a water-soluble monomeric protein. Biochem. Biophys. Res. Comm., *1:* 133–137.

Chance, B. 1954 Spectrophotometry of intracellular respiratory pigments. Sciences, *120:* 767–775.

Chance, B., and M. Baltscheffsky 1958 Spectroscopic effects of adenosine diphosphate upon the respiratory pigments of rat-heart muscle sarcosomes. Biochem. J., *68:* 283–295.

Chance, B., and B. Sacktor 1958 Respiratory metabolism of insect flight muscle. II. Kinetics of respiratory enzymes in flight muscle sarcosomes. Arch. Biochem. Biophys., *76:* 509–531.

Chance, B., and G. R. Williams 1955 Respiratory enzymes in oxidative phosphorylation. II. Difference spectra. J. Biol. Chem., *217:* 395–407.

——— 1956 The respiratory chain and oxidative phosphorylation. Adv. Enzymol., *17:* 65–130.

Chance, B., and T. Yonetani 1959 Reactions of cytochrome oxidase. Fed. Proc., *18:* 202.

Estabrook, R. W. 1956 The low temperature spectra of hemoproteins. I. Apparatus and its application to a study of cytochrome c. J. Biol. Chem., *223:* 781–794.

Estabrook, R. W., and B. Mackler 1957 The low temperature spectra of hemoproteins. II. Cytochromes of heart muscle preparations. Ibid., *224:* 637–648.

Ghiretti-Magaldi, A., A. Giuditta and F. Ghiretti 1957 A study of the cytochromes of *Octopus vulgaris* Lam. Biochem. J., *66:* 303–307.

——— 1958 Pathways of terminal respiration in marine invertebrates. I. The respiratory system in cephalopods. J. Cell. and Comp. Physiol., *52:* 389–430.

Ghiretti, F., A. Ghiretti-Magaldi and L. Tosi 1959 Pathways of terminal respiration in marine invertebrates. II. The cytochrome system of *Aplysia*. J. Gen. Physiol., *42:* 1185–1205.

Green, D. E. 1959 Mitochondrial structure and function. Subcellular particles. Washington, D. C., pp. 84–103.

Gumbmann, M., W. D. Brown and A. L. Tappel 1958 Intermediary metabolism of fishes and other aquatic animals. Special Sci. Rept. Fisheries No. 288, Washington, D. C., pp. 1–51.

Kawai, K. 1958 Cytochrome system in oyster tissues. Nature, *181:* 1468.

Ramirez, J. 1959 Oxidation-reduction changes of cytochromes following stimulation of amphibian cardiac muscle. J. Physiol., *147:* 14-32.

Phloretin, Phloridzin and Glucose Inhibition of a Phosphatase[1] on the Stromata of Rabbit Erythrocytes[2]

PHILIP C. LARIS, GORDON NOVINGER AND JOHN CALAPRICE
Department of Biological Sciences, University of California at Santa Barbara, Goleta, California.

Several authors have proposed "carrier mechanisms" for sugar transport in human erythrocytes (Le Fevre, '54; Wilbrandt, '54; Widdas, '52) and in rabbit erythrocytes (Park et al., '56). Although the proposed site of interaction of membrane and sugar has not been identified, some of its properties are known, i.e., phloretin and phloridzin act as competitors with sugar for the carrier in human erythrocytes according to Le Fevre ('54). While studying the properties of a phosphatase of rabbit erythrocyte stromata with adenosine triphosphate as substrate, it was determined that phloretin, phloridzin, and glucose inhibit the phosphatase activity. There is a similarity between the inhibitory capacities of these substances as measured in these investigations and their relative affinities for the "carrier" as measured by Le Fevre ('54).

METHODS

Preparation of stromata. Blood was obtained from rabbits by heart puncture and defibrinated with glass beads. The cells were washed three times with approximately 10 times their volume of 1% sodium chloride solution. The buffy coat and top layers of red cells were discarded. The hematocrit was determined by centrifuging a sample at 3000 rpm for 30 minutes. The cell suspension was then adjusted to a hematocrit of 40%. The erythrocytes were lysed by alternate freezing and thawing 4 times with dry ice and a water bath at 37°C. The hemolysate was centrifuged for 30 minutes at 6000 rpm at 4°C in a Serval refrigerated centrifuge after addition of 15 times its volume of 0.9% NaCl. The stromata were washed 4 times in this manner and the final volume adjusted to that of the initial whole hemolysate (40% hematocrit). Stromata were stored at − 12°C and were used one or two days after preparation.

Determination of enzymatic activty. The enzymatic activity was studied in 3 ml of a solution containing 0.035 M glycylglycine adjusted to pH 7.40 ± 0.02 with NaOH, 0.0067 M $MgCl_2$, 0.1 M NaCl, 0.1 ml stromata or 0.1 ml 0.9% NaCl and 0.413 mM adenosine triphosphate (sodium salt). All components except stromata and substrate were mixed first. The stromata were added to this mixture and the tubes (handled and timed individually) were placed in a water bath at 37°C for 10 minutes (as in Herbert, '56). After this preliminary incubation, the substrate was added. The reaction was stopped by the addition of 6 ml of cold 12% trichloracetic acid solution. Inorganic phosphate was determined by the method of Fiske and SubbaRow ('25). All determinations were made in duplicate.

RESULTS

The rate of liberation of inorganic phosphate from adenosine triphosphate was constant for the 32 minute time period studied (fig. 1) and hence experiments with inhibitors were carried out for 20 to 30 minutes. In figure 2 the percentage inhibition is shown as a function of phloretin, phloridzin, and glucose concentrations. Phloretin is relatively insoluble in water and was first dissolved in ethanol.

---

[1] The specificity of this enzyme and its mechanism of action have not been determined. It shall be referred to as phosphatase throughout this study.

[2] This work was supported by funds from the Research Committe of the University of California at Santa Barbara.

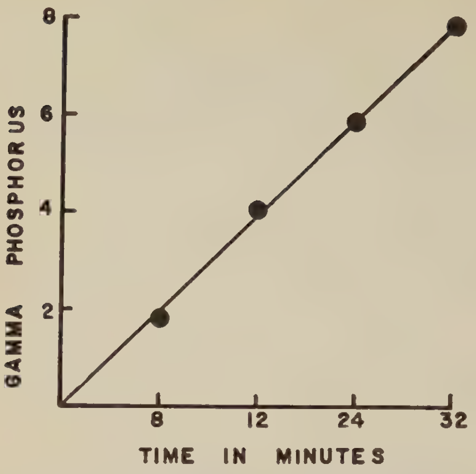


Fig. 1 The liberation of inorganic phosphate from adenosine triphosphate (0.413 mM/1) by 0.1 ml rabbit erythrocyte stromata (recovered from 0.1 ml intact erythrocytes of 40% hematocrit).

The concentration of ethanol in the test solutions was 0.25% and ethanol did not affect the enzymatic activity in this amount.

DISCUSSION

The relative affinities of phloretin, phloridzin, and glucose for the "sugar carrier" have been calculated by Le Fevre ('54) for the human erythrocyte. Of the three, phloretin has the greatest affinity (1800 times that of sugar) and phloridzin is intermediate (70 times that of sugar). The concentrations of these substances producing 50% inhibition in the present investigations are 3.5 × 10^{-5} M phloretin, 6 × 10^{-4} M phloridzin, and 6 × 10^{-2} M glucose. To produce the same effect (50% inhibition), the concentrations of glucose must be 1700 times as great as phloretin and 100 times as great as phloridzin. The affinities for the carrier and the affinities for the enzyme are of the same order of magnitude. Penetration of sugar through the membrane may require attachment to sites on this enzyme. It is suggested that the enzyme molecules are "non-mobile components" of the membrane which serve as a series of sugar adsorbing sites through the membrane (the polar "creep" hypothesis of Bowyer and Widdas, '56). Phosphatases are present at the surface in proximal kidney tubule cells and intestinal mucosa (Danielli, '52) and in human erythrocytes (Herbert, '56) and could have a similar function in these locations.

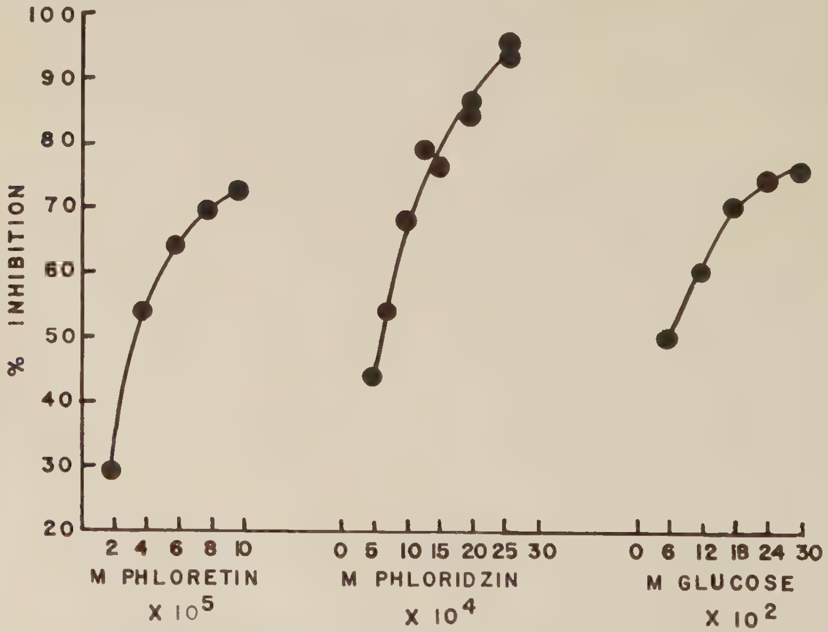


Fig. 2 The percentage inhibition of enzymatic activity in the presence of phloretin, phloridzin and glucose. Note changes in abscissa.

Adenosine triphosphate does not appear to be necessary for sugar penetration in rabbit or human erythrocytes, for entrance will occur while glycolysis is inhibited with iodoacetic acid or sodium fluoride (Le Fevre, '54; Laris, '58). Hydrolysis of adenosine triphosphate would not be necessary according to the mechanism presented here and, in fact, adenosine triphosphate could slow down penetration by competing with sugar. In this connection it is interesting to note that Wilbrandt ('54) has reported that adenosine triphosphate does inhibit glucose penetration in human erythrocytes.

Phloridzin and phloretin also inhibit movement of phosphate (P^{32}) into human red cells (Harris and Prankerd, '58). The inhibition of phosphate uptake could be accomplished through the inhibition of this enzyme since Gourley ('52) has proposed that penetration of phosphate involves the formation and hydrolysis of adenosine triphosphate.

SUMMARY

Phloretin, phloridzin, and glucose inhibit the hydrolysis of adenosine triphosphate by an enzyme on the rabbit erythrocyte stroma. Implication of these inhibitory effects are discussed in relation to sugar permeability.

LITERATURE CITED

Bowyer, F., and W. F. Widdas 1958 The action of inhibitors on the facilitated hexose transfer system in erythrocytes. J. Physiol., *141:* 219–232.

Danielli, J. F. 1952 Structural factors in cell permeability and secretion. Symp. Soc. Exp. Biol., *VI:* 1–15.

Fiske, C. H., and Y. SubbaRow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375–400.

Gourley, D. R. H. 1952 The role of adenosine triphosphate in the transport of phosphate in the human erythrocyte. Arch. Biochem. Biophys., *40:* 1–12.

Harris, E. J., and T. A. J. Prankerd 1958 Phloridzin and red cell phosphate turnover. Experientia, *14:* 249.

Herbert, E. 1956 A study of the liberation of orthophosphate from adenosine triphosphate by the stromata of human erythrocytes. J. Cell. and Comp. Physiol., *47:* 11–36.

Laris, P. C. 1958 Permeability and utilization of glucose in mammalian erythrocytes. Ibid., *51:* 273–307.

Le Fevre, P. 1954 The evidence for active transport of monosaccharides across the red cell membrane. Symp. Soc. Exp. Biol., *VIII:* 118–135.

Park, C. R., R. L. Post, C. F. Kolman, J. H. Wright, L. H. Johnson and H. E. Morgan 1956 The transport of glucose and other sugars across cell membrane and the effect of insulin. Ciba Foundation Colloquia on Endocrinology, Little, Brown and Co., Boston, *9:* 240–260.

Widdas, W. F., 1954 Facilitated transfer of hexoses across the human erythrocyte membrane. J. Physiol., *125:* 163–180.

Wilbrandt, W. 1954 Secretion and transport of non-electrolytes. Symp. Soc. Exp. Biol., *VIII:* 136–162.

Behaviors of Membrane Potential in the Cockroach Giant Axons Poisoned by DDT[1]

TOSHIO NARAHASHI AND TERUO YAMASAKI
Laboratory of Applied Entomology, Faculty of Agriculture, University of Tokyo, Tokyo, Japan

The negative after-potential of the cockroach giant axon is greatly augmented and prolonged by the treatment with DDT. When the DDT-poisoned axon is in contact with potassium-free Ringer's solution, the negative after-potential is further augmented showing a plateau during its declining phase (Narahashi and Yamasaki, '60). Action potentials having a plateau during their declining phase are well known in various nerves and muscles, e.g., normal cardiac muscle (Cranefield and Hoffman, '58a; Weidmann, '57), and squid giant axons, nerve cells of *Onchidium* and frog spinal ganglion cells each treated with tetraethylammonium (TEA) (Tasaki and Hagiwara, '57; Hagiwara and Saito, '59; Koketsu, Cerf, and Nishi, '59). Attempts were made to explain these plateaus in terms of ionic conductances (Shanes, '58b; Weidmann, '57).

The increase and prolongation of the negative after-potential by DDT have been demonstrated to be explicable by a delay and partial suppression of the rise in potassium conductance which accelerates the falling phase of the action potential (Narahashi and Yamasaki, '60). However, since that study was mainly concerned not with the plateau but with the increased negative after-potential in the initial stages of DDT poisoning, the mechanism of the plateau formation and behaviors of the membrane potential in the later stages of DDT poisoning remain to be seen. Analyses are therefore extended to the present study, which is mainly concerned with behaviors of the membrane potential in the DDT-poisoned cockroach axons having the ability to give rise to action potentials with a plateau.

METHODS

The giant axons of the cockroach, *Periplaneta americana* L., were used throughout the experiments. The methods of stimulation, recording, perfusion, Ringer's solution, and DDT application were the same as those described previously (Narahashi and Yamasaki, '60). Concentration of *p,p'*-DDT was always 10^{-4} M. The DDT-poisoned axons were further treated with potassium-free Ringer's solution unless otherwise stated. Potassium-free Ringer's solution was prepared by replacing K by Na. All the experiments were conducted at room temperatures ranging from 16.5 to 26.5°C.

As described previously, the negative after-potential increases progressively after treatment with DDT, attains a maximum height, and eventually begins to decrease (Narahashi and Yamasaki, '60). There is in the course of this poisoning a brief period during which repetitive responses can be induced by a single shock. The time course of DDT poisoning can be divided into 4 stages: The period from the beginning of the increase in negative after-potential to the end of repetition (stage I); from the end of repetition to the maximum negative after-potential (stage II); from the beginning of slowing of the rate of fall of the spike potential to the beginning of decrease in negative after-potential (stage III); the period of marked decrease in negative after-potential (stage IV) (Narahashi and Yamasaki, '60). The present experiments were made on the DDT-poisoned axons in the stages from the end of the stage II to the stage IV, unless otherwise stated.

RESULTS

Lowering of critical depolarization and threshold current

The potential at the inflection point between the catelectrotonic potential and the action potential when the threshold current was applied was taken as a measure of critical depolarization. During the period from the end of stage II to the stage III the critical depolarization was greatly reduced. Example of such a record is shown in the bottom of figure 1. In this particular case the critical depolarization amounted to 7 mv. The values were usually of the order of 5–14 mv, as against the normal mean value of 22 mv (Yamasaki and Narahashi, '59b). The threshold current was also reduced; this value varied considerably from fiber to fiber, being 1.4–1.8 × 10^{-9} a. in one case and 7.5–25.6 × 10^{-9} a. in another case, as against the normal mean value of 44.2 × 10^{-9} a. (Yamasaki and Narahashi, '59b). Since the resting potential undergoes little change (Narahashi and Yamasaki, '60), it can be stated that the membrane potential at which the action potential is fired is raised by DDT.

---

[1] Studies on the mechanism of action of insecticides. XX.

In the last stage of poisoning (stage IV), the critical depolarization was restored toward the normal level.

Anode break excitation

Anode break excitations were easily elicited in the DDT-poisoned axons during the stages II, III and IV. The median record of figure 1 is a superimposed photograph of 4 responses induced by the same intensity of anodal current. Two of them failed to fire propagated action potential, and only produced small graded response which will be described later. Small hyperpolarization was sufficient for anode break excitation. Figure 2 clearly indicates that the current that failed to produce action potential when applied cathodal direction was effective in producing action potential when applied anodal direction.

Graded response

In the giant axons of the cockroach, graded response is invariably observed with subthreshold cathodal current (Yamasaki and Narahashi, '59b). When poisoned with DDT, these became much more conspicuous and were also observed on break of anodal current. This effect of DDT became more marked with the advance of time after treatment.

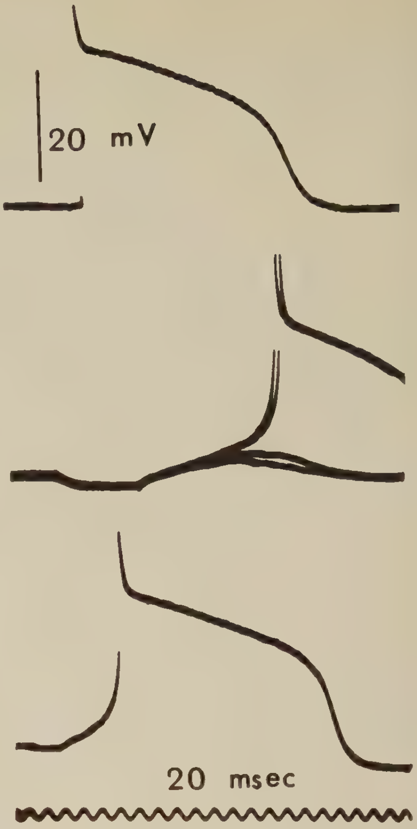


Fig. 1 Action potentials induced by a singl shock applied to the end of the nerve prepara tion (top record), by pulses of anodal curren applied to the point of recording (median super imposed record), and by a pulse of cathodal cur rent applied to the point of recording (botto record) in potassium-free DDT solution; 122–13 minutes after treatment with DDT. Spike po tentials are too large to be recorded. Tempera ture 26.5°C.

Figure 2 shows the graded responses a induced both by cathodal and by anoda currents of various intensities. The grade responses, being much prolonged, could b seen with very weak cathodal and anoda currents. A depolarization or hyperpolari zation as small as about 3 mv was suffici ent for the graded response to be induced This value is much smaller than the de polarization needed for the graded respons in the normal axons which is of the orde of about 10 mv (Yamasaki and Nara hashi, '59b). The height of the graded re sponse increased with raising the curren intensity, the increase being more marke

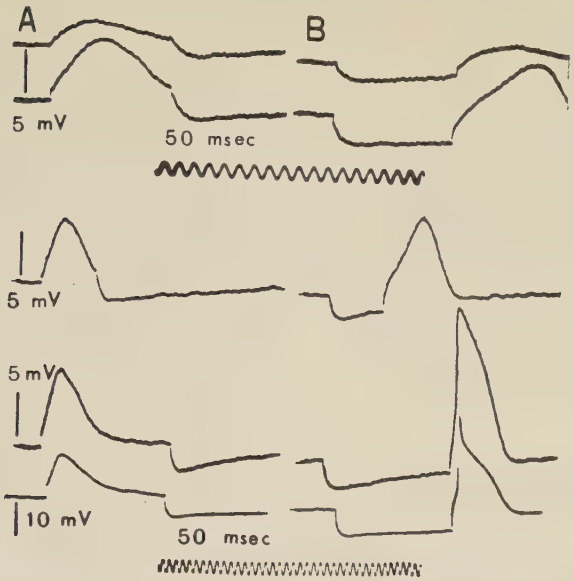


Fig. 2 Graded responses on make of cathodal current (column A) and on break of anodal current (column B) in potassium-free DDT solution; 290 minutes after treatment with DDT. The intensity of current is raised from the top to the bottom records, and is the same in the same row of A and B. A spike potential is induced in the bottom record of B, but is too large to be recorded. Temperature 18°C.

vith anodal current than with cathodal urrent. Its rate of rise also increased with aising the current intensity, whereas the ate of fall increased to a lesser extent. The full size graded response, when atained a critical level, gave rise to a spike otential which declined with a similar ime course as that of the graded response.

Thus, the differences in graded response etween the normal and the DDT-poisoned xons can be summarized as follows: (a) he graded responses are observed in the oisoned axons with much weaker current han in the normal axons; (b) they can be bserved with both cathodal and anodal urrents in the poisoned axons, whereas nodal current is ineffective in the nornal axons; (c) the time course is much nore prolonged in the poisoned axons than n the normal axons.

The graded responses observed with the DT-poisoned axons well resemble those bserved with cardiac fibers in normal nd in low calcium conditions in that the ize depends on the stimulus intensity, hat the rate of rise is slow and the shape s rounded with weak stimuli, and that he shape resembles the action potential vith stronger stimuli (Hoffman and Suckling, '56; Hoffman, Kao, and Suckling, '57; Kao and Hoffman, '58).

Spontaneous excitation

Some of the DDT-poisoned axons showing low threshold discharged propagated impulses spontaneously. Records of the action potential induced spontaneously and by a single stimulus are illustrated by figure 3. The artificially induced action potential (top record) arose abruptly from the resting level, whereas the spontaneous one (median and bottom records) was preceded by a gradually rising slow potential. No difference in configuration was observed between them except for the prepotential. On some occasions the graded potentials similar to those induced by polarizing current were observed without any stimulation. This fact may imply that as in the artificially induced graded potentials the spontaneous graded potentials give rise to propagated action potentials when attaining a certain critical level.

It is interesting to note that the prepotentials are usually seen in cardiac and skeletal muscle fibers when spontaneously active (Bülbring, Holman, and Lüllman,

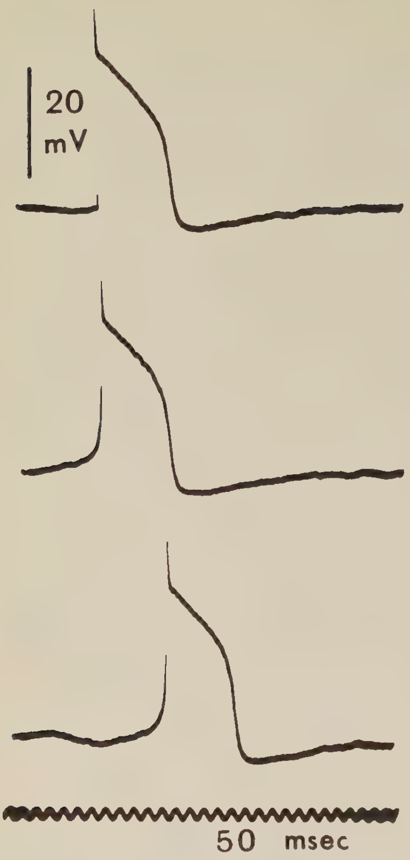


Fig. 3 Action potentials induced by a single shock applied to the end of the nerve preparation (top record), and induced spontaneously (median and bottom records) in potassium-free DDT solution; 145–152 minutes after treatment with DDT. Spike potentials are too large to be recorded. Temperature 26.5°C.

'56; Hoffman and Suckling, '56; Weidmann, '51).

Abolition of the action potential by anodal current

Figure 4 is the record showing the effect of brief pulses of anodal current with various intensities delivered at a fixed instant during the plateau of the action potential. On cessation of weak anodal current, the membrane potential returned nearly to the potential level which would have been attained if the anodal current had not been delivered (second record in A). However, with raising the current intensity, the membrane potential, on cessation of the current, attained a new level which was lower than that expected from the normal declining phase (third record in A). With further increase in current intensity, the membrane potential was suddenly brought to the resting level on cessation of the current (bottom record in A). When the intensity of current was raised beyond this level, a graded response was set up on cessation of the current (top and median records in B). A propagated action potential was fired from the graded response when the current intensity was further raised (bottom record in B).

The present experiments were made with capillary microelectrodes inserted transversely into the axon. Under this condition any potential changes occurring at the point of impalement would spread electrotonically along the axon. Likewise, the potential changes originating in the neighborhood of the impalement would partly be recorded. For these reasons it is impossible to estimate the absolute value of the critical potential for abolition or abolition threshold by the present technique. However, the relative value for the threshold during the time course of the plateau could be estimated by applying various intensities of anodal current at several instants of the plateau. Figure 5 shows such records. It will be seen that greater hyperpolarization was needed for abolishing the plateau when applied to earlier phase.

These changes in abolition threshold in the DDT-poisoned axons are in agreement with those estimated in the single node of the bullfrog or the toad (Tasaki, '56) and in the TEA-treated squid giant axons (Tasaki and Hagiwara, '57).

Abolition of the action potential by anodal current is also well known in heart muscle. Cranefield and Hoffman ('58b) pointed out the difference between cardiac muscle and Purkinje fibers in response to anodal current: In cardiac muscle fibers weak anodal current applied during the plateau causes an abolition, whereas stronger anodal current causes a break excitation; in Purkinje fibers the reverse relation is held, weak current causing a break excitation and stronger current causing an abolition.

Both types are actually observed in the DDT-poisoned axons, the former type be-

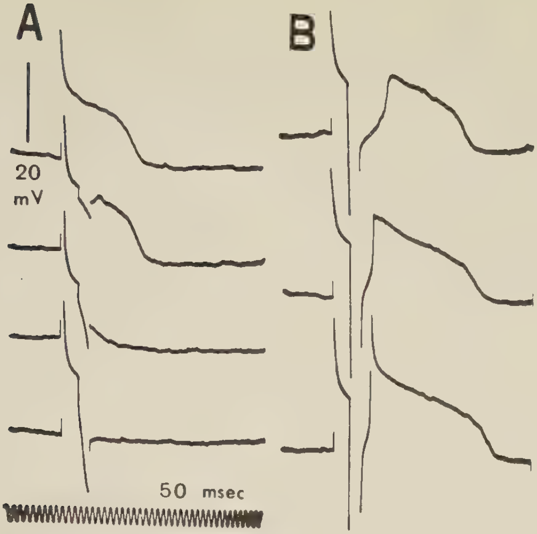


Fig. 4 Abolition of the plateau by anodal current and anode break excitation in potassium-free DDT solution; 165 minutes after treatment with DDT. The anodal current is delivered at a fixed instant during the plateau and increased in intensity from the second record in column A to the bottom record in column B. The top record in A is an action potential without superimposed anodal pulse. Spike potentials are too large to be recorded. Temperature 16.5°C.

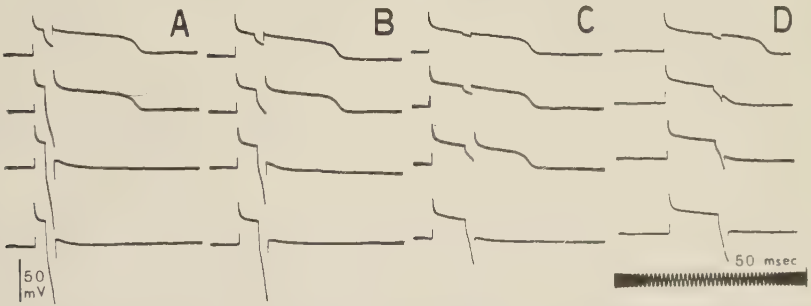


Fig. 5 Changes in abolition threshold during the plateau in potassium-free DDT solution; 145 minutes after treatment with DDT. In each column, anodal current is delivered at a fixed instant and increased in intensity from the top to the bottom records. The current intensity is not the same in the same row of each column. Spike potentials are too large to be recorded. Temperature 16.5°C.

ing seen in figure 4 and the latter in figure 5. These two types may be explained as follows: The abolition threshold is lowered toward the end of the plateau. The threshold for anode break excitation also changes during the plateau, as will be described in the subsequent section. These two threshold values would determine the order by which these two phenomena occurred successively as stimuli with increasing intensities were applied at any moments of the plateau.

Refractoriness for the spike height

Brief cathodal and anodal currents were delivered at various instants during the plateau. A series of records is illustrated by figure 6. The intensity of cathodal current was approximately half of that of anodal current in this case. The anodal current used was ineffective in eliciting break response in the resting membrane (second record in A). Although it produced only a small graded response when

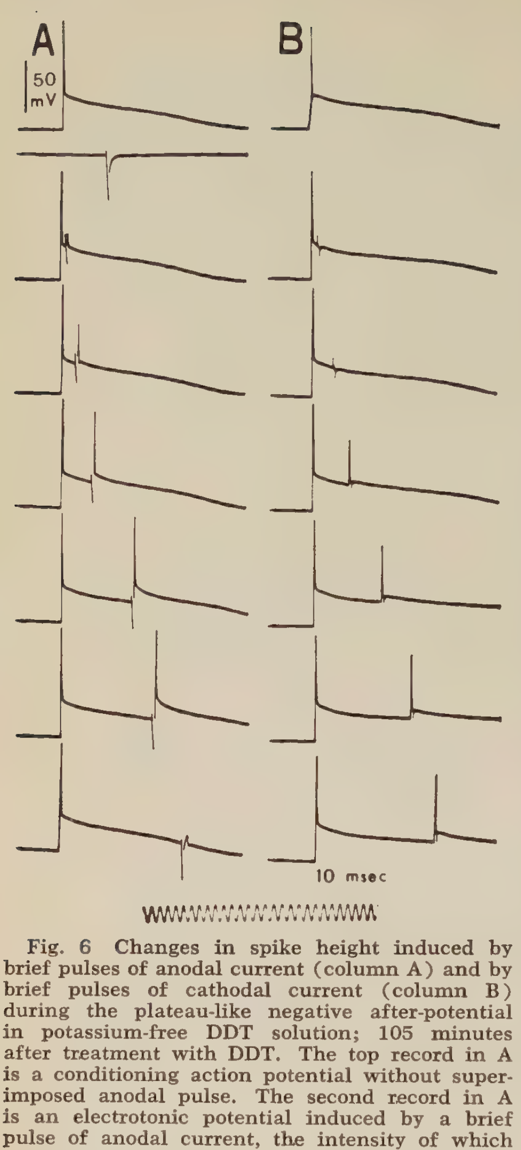


Fig. 6 Changes in spike height induced by brief pulses of anodal current (column A) and by brief pulses of cathodal current (column B) during the plateau-like negative after-potential in potassium-free DDT solution; 105 minutes after treatment with DDT. The top record in A is a conditioning action potential without superimposed anodal pulse. The second record in A is an electrotonic potential induced by a brief pulse of anodal current, the intensity of which is kept constant to the bottom record. The top record in B is a testing action potential induced by a brief pulse of cathodal current without conditioning stimulus, the intensity of which is kept constant to the bottom record. Temperature 16.5°C.

delivered immediately after the spike (third record in A), it became effective in producing large spike potential when delivered at later instants (4th through 7th records in A). However, it became again ineffective near the terminal phase of the plateau (bottom record in A). The cathodal current, though ineffective immediately after the spike (second and third records in B), increased its effectiveness more and more toward the end of the plateau (4th through 7th records in B).

Refractoriness for the action potential duration

The second action potential induced during the plateau of the first action potential was depressed not only in height but also in duration. Records showing this are illustrated by figure 7, in which both conditioning and testing stimuli were applied to the end of the nerve preparation by external electrodes. When a testing stimulus was applied immediately after the spike, an action potential with small amplitude and short duration was elicited which was undoubtedly conductive (top record in A). With lengthening the stimulus interval, the spike height of the testing impulse grew more and more until it attained a nearly normal level (second through bottom records in A). This recovery is in accordance with that tested by brief cathodal currents as described in the foregoing section.

The duration of the action potential, however, remained curtailed even when the spike height was restored to its full size. With further lengthening the stimulus interval, the action potential duration was restored progressively, but the interval as long as about one second was still insufficient for the duration to be restored completely (B). These facts may imply that the refractoriness for the duration of the action potential is at least in part governed by some factors different from those which govern the refractoriness for the spike height.

Abolition of refractoriness by anodal current

An anodal current delivered during the plateau could abolish not only action potential but also refractoriness, the records being illustrated by figure 8. Upper tracings in each column, which were induced by two stimuli applied through external electrodes, show that the second action potential was curtailed effectively. However, when the testing stimulus was preceded by a brief pulse of anodal current sufficient for abolishing the plateau, the

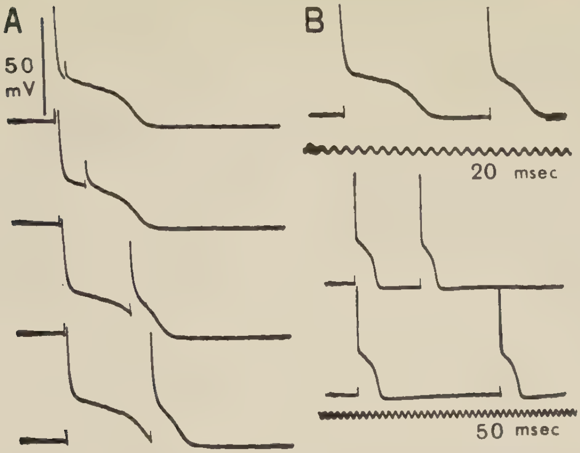


Fig. 7 Prolonged refractoriness for the action potential duration in potassium-free DDT solution; 277–287 minutes after treatment with DDT. Supramaximal conditioning and testing stimuli are applied to the end of the nerve preparation at varying intervals. The upper time marker in column B applies both to column A and to the top record in B. Temperature 26.5°C.

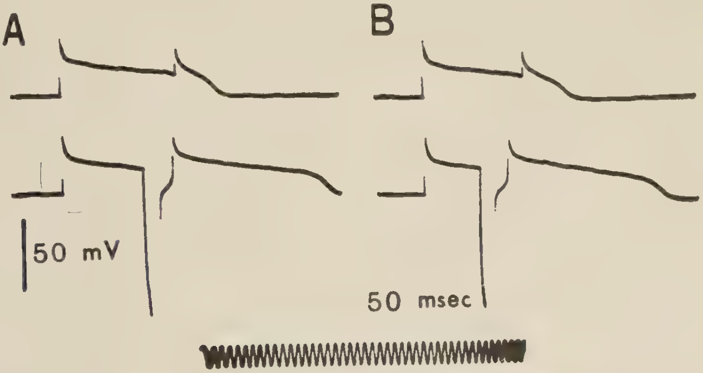


Fig. 8 Abolition of refractoriness by a pulse of anodal current in potassium-free DDT solution; 155 minutes after treatment with DDT. The upper record in each column is obtained by applying supramaximal conditioning and testing stimuli to the end of the nerve preparation. The lower record is obtained by delivering a pulse of strong anodal current immediately before the testing stimulus. Spike potentials are too large to be recorded. Temperature 16.5°C.

second action potential was restored to its full duration (lower tracings).

The abolition of refractoriness is in good agreement with that observed with the TEA-treated squid axons (Tasaki and Hagiwara, '57).

Membrane resistance during the prolonged negative after-potential and the plateau

Since polarization and recording are made at the same point of the axon, the magnitudes of the electrotonic potentials induced by the same currents at various instants during the plateau are indicative of relative changes of the membrane resistance (see Hodgkin and Rushton, '46).

Figure 9 shows a series of records taken from the poisoned axon whose negative after-potential does not yet attain the maximum height. Records from the poisoned axon which develops the full size plateau are shown in figure 10. Further, in figure 6A are seen records from the axon which develops the plateau-like negative after-potential.

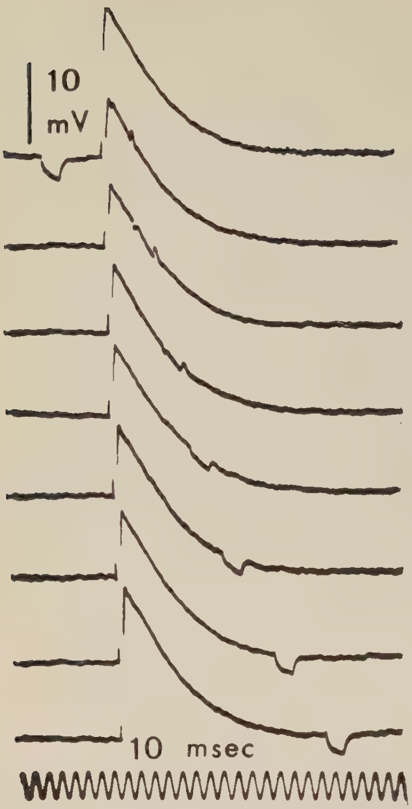


Fig. 9 Brief anelectrotonic potentials induced at various instants during the declining phase of the negative after-potential augmented by potassium-free DDT solution; 65 minutes after treatment with DDT (initial stage of poisoning). The intensity of anodal current is the same in all records. Spike potentials are too large to be recorded. Temperature 16.5°C.

Relative membrane resistances during the negative after-potential and plateau were calculated from these figures, and are illustrated by figure 11. It will be seen that, in the moderately increased negative after-potential, the membrane resistance was very low immediately after the spike and rised rapidly with the repolarization toward the resting level (A). In the case of the plateau, the membrane resistance was reduced to a much lesser extent immediately after the spike, and rose more slowly toward the end of the plateau (B and C).

The changes in membrane resistance during the augmented negative after-potential and the plateau are almost the same as those described in Purkinje fibers (Weidmann, '51) and TEA-treated nerve cells and axons (Koketsu, Cerf, and Nishi, '59; Tasaki and Hagiwara, '57).

Effect of electrotonus on the prolonged negative after-potential

Changes in the DDT-induced negative after-potential by electrotonus are shown in figure 12. The records were taken in the Ringer's solution containing DDT, so that no marked plateau phase was apparent. It can be seen that the height of the negative after-potential was further augmented by anelectrotonus and depressed by catelectrotonus (second and third records). With greater hyperpolarization, a plateau became apparent during the declining phase of the greatly augmented negative after-potential. Thus, the effect of electrotonus on the height of the negative after-potential is at least qualitatively similar to that observed in the normal unpoisoned axons (Yamasaki and Narahashi, '59b).

The duration of the action potential was effectively shortened by catelectrotonus, whereas it was only slightly lengthened by anelectrotonus.

DISCUSSION

In the later stages of DDT poisoning (stages III and IV), the falling phase of

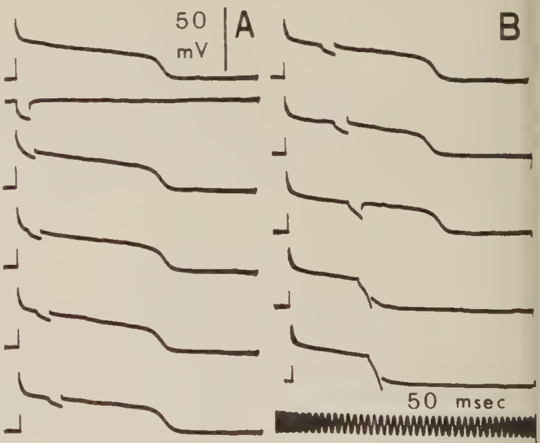


Fig. 10 Brief anelectrotonic potentials induced at various instants during the plateau in potassium-free DDT solution; 136–140 minutes after treatment with DDT. The intensity of anodal current is the same in all records. Spike potentials are too large to be recorded. Temperature 16.5°C.

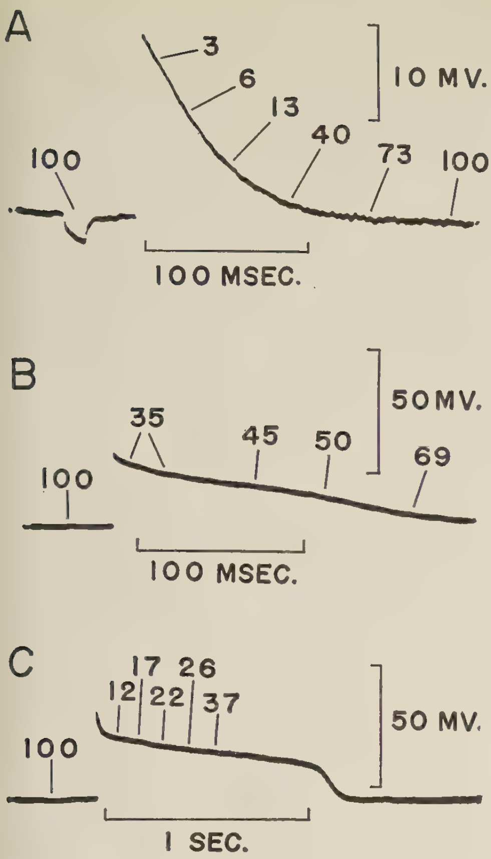


Fig. 11 Changes in relative membrane resistance during the negative after-potential augmented by potassium-free DDT solution. The membrane resistance of the resting membrane is taken as 100. The three records are taken from the same axon. A, 65 minutes after treatment with DDT, the same as the top record in figure 9; B, 105 minutes after DDT, the same as the top record in figure 6A; C, 136 minutes after DDT, the same as the top record in figure 10A.

the spike is greatly slowed down. The spike height induced by the testing cathodal current during the plateau is lower than normal and grows progressively toward the end of the plateau. Anode break excitations are easily elicited during the plateau, and the spike height may be higher than that induced by cathodal current. All these facts are in support of the view that there is an incomplete inactivation during the plateau; small spike height during the plateau may be due to the incomplete inactivation, and improvement of it by anodal current may be accounted for by a removal of inactivation. The removal of inactivation by anodal current also seems to be responsible for the disappearance of refractoriness after abolition.

It should be recalled that anode break excitations cannot be observed in the normal cockroach giant axons (Yamasaki and Narahashi, '59b). There is general agreement that nerves and muscles, though they do not respond with break of anodal current in normal good conditions, become excitable on the break of anodal current when they are in abnormal conditions such as slight depolarization (Frankenhaeuser and Widén, '56; Ichioka and Konishi, '57), low concentrations of calcium (Frankenhaeuser, '57; Hoffman and Suckling, '56),

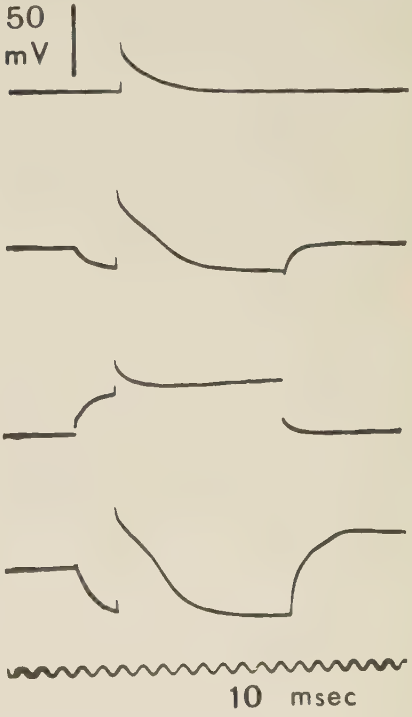


Fig. 12 Effect of electrotonus on the negative after-potential augmented by DDT; 69–73 minutes after treatment with DDT. Top record, the negative after-potential induced in the resting membrane; second record, the hyperpolarized membrane; third record, the depolarized membrane, the intensity of cathodal current is the same as that of the anodal current used in the second record; bottom record, the hyperpolarized membrane, the current intensity is greater than that in the third record. Spike potentials are too large to be recorded. Temperature 25°C.

and under the influence of cocaine (Weidmann, '55). All these conditions favor the explanation put forward by Hodgkin and Huxley ('52) that the anode break excitation is brought about because anodal polarization decreases potassium conductance and removes inactiviation.

However, there are some difficulties in applying this explanation to the present case: Unlike the plateau phase, no inactivation seems to occur in the resting state of the DDT-poisoned axons, because the resting potential is not lowered and the full size spike potentials are able to be elicited by cathodal current. The mechanism for anode break excitation in the resting DDT-poisoned axons remains to be seen.

It has been demonstrated in our previous paper that the increase and prolongation of the negative after-potential in the initial stages of DDT poisoning (stages I and II) are explicable by a delay and suppression of the rise in potassium conductance (G_K) which accelerates the falling phase of the action potential. The inactivation process which lowers sodium conductance (G_{Na}) once raised during the rising phase of the action potential was thought to be unaffected by DDT at least in these initial stages (Narahashi and Yamasaki, '60). In the later stages (stages III and IV), however, this process can be assumed to be inhibited to some extent as mentioned above. It follows that, G_{Na}, which declines after the spike more slowly than normal, approaches and finally equals G_K, the rise of which is now greatly delayed and suppressed. Further, if it is assumed that the membrane potential is governed both by the concentration gradients of sodium and potassium ions between inside and outside of the membrane and by membrane permeabilities to these ions, we have the following equation at 18°C (see Shanes, '58a):

$$E_m = 58 \log \frac{P_K(K)_i + P_{Na}(Na)_i}{P_K(K)_o + P_{Na}(Na)_o},$$

where E_m is membrane potential, P_K and P_{Na} are membrane permeabilities to potassium and sodium, $(K)_i$ and $(Na)_i$ are intracellular concentrations of potassium and sodium, and $(K)_o$ and $(Na)_o$ are extracellular concentrations. The values for $(K)_i$ and $(Na)_i$ in the cockroach nerve are estimated as 140 and 84 mM respectively (Tobias, '48). $(K)_o$ and $(Na)_o$ are 3.1 and 214 mM respectively in the present experiments. Thus, in the condition satisfying $P_K = P_{Na}$ or $G_K = N_{Na}$, E_m is nearly zero. The initial height of the plateau is of the order of 30 mv or more. Such potential levels would be maintained if G_K were somewhat higher than G_{Na}. This condition would be brought about either (a) by a fall of G_{Na} keeping G_K unchanged or (b) by a fall of G_{Na} to a lesser extent accompanied by a small rise in G_K. If, thereafter, the ratio G_K/G_{Na} were raised only slowly, the membrane potential would decline slowly forming a plateau. The fact that membrane conductance is of appreciable value immediately after the spike and increases slowly during the plateau seems to be rather in favor of the view (a) above, though the possibility of (b) cannot be excluded completely.

There are three possible explanations for the ionic mechanism of the repolarization phase which curtails the plateau: (a) Fall of G_{Na} to the resting level; (b) rise in external potassium concentration near the membrane; (c) rise in G_K. Since G_{Na} was thought not to be lowered completely to the resting level during the plateau, its fall would cause repolarization. However, this does not explain the positive overshoot occurring after the plateau, which excludes the possibility of (a) above. If a small rise in G_K occurred during the plateau, there would be a slight exit of potassium ions. The accumulation of released potassium would shorten the plateau, because the increase in external potassium concentration was shown to do so (Narahashi and Yamasaki, '60). However, it is difficult to explain the sudden repolarization by such a graded rise in potassium concentration. This excludes the possibility (b) above. Rise in G_K may occur when the plateau phase reaches a certain level, causing a repolarization. The presence of the positive overshoot after the plateau is compatible with this view, because the equilibrium potential for potassium ions is somewhat larger than the resting potential (Yamasaki and Narahashi, '59a). Such a delayed onset of the rise in G_K is also expected from the previous finding that the delayed rectification is further delayed and suppressed by DDT (Narahashi and Yamasaki, '60).

Summarizing the ionic mechanism for he production of the action potental showng a plateau under the influence of DDT, he sequence of events appears to be (a) an ncrease in G_{Na} which causes an overshoot (the spike peak); (b) an incomplete inactivation or slow decline of G_{Na}, resulting n G_{Na} being somewhat lower than G_K vhich undergoes little change (the plateau); (c) when the level of the plateau eaches a certain level, G_K temporarily increases causing an abrupt repolarization followed by a positive overshoot.

This explanation is essentially similar to those proposed for the plateaus of heart muscle and TEA-treated squid axons by Weidmann ('57) and Shanes ('58b). It is interesting to note that the action potential from the DDT-poisoned cockroach giant axons, when further treated with potassium-free solution, resembles those from heart muscle and TEA-treated squid axons not only in the shape but also in the mechanism of production.

There are resemblances also in behaviors of the membrane potential. These behaviors are (a) spontaneous excitation, (b) anode break excitation during the plateau, (c) graded response with variable sizes depending on the stimulus intensity, and (d) abolition of the plateau by anodal current.

No sufficient account is available for the mechanism of abolition of the plateau by anodal current. It seems reasonable to assume that there is a labile equilibrium state of the membrane potential as suggested by Weidmann ('57) for the plateau of cardiac muscle. When the repolarization does not exceed this level, the membrane potential, on cessation of the current, is brought back to the plateau level which is in a semistable equilibrium state. However, when repolarized beyond this level, the membrane potential is brought to the resting level which is in a stable equilibrium state.

SUMMARY

1. Behaviors of the membrane potential have been analyzed on the cockroach giant axons treated with potassium-free DDT solution which generate the prolonged action potentials showing a plateau in their repolarizing phase.

2. The critical depolarization for the spike was greatly reduced.

3. Anode break excitations were easily elicited both in the resting membrane and during the plateau.

4. Graded responses with long durations were induced on make of cathodal current and on break of anodal current. Their amplitudes were variable and depended on the stimulus intensity.

5. Action potentials and graded potentials were elicited spontaneously in some cases. The action potential was always preceded by a slowly depolarizing potential.

6. The plateau could be abolished by a brief pulse of anodal current if of sufficient intensity. The abolition threshold was highest at the beginning of the plateau and lowered toward the end of the plateau.

7. The height and duration of the second action potential induced during the plateau were reduced. Refractoriness for the action potential duration was markedly prolonged beyond that for the spike height. These refractorinesses were removed by the abolition of the plateau.

8. Membrane resistance immediately after the spike, though lower than that in the resting state, was in appreciable value, and rose slowly toward the end of the plateau.

9. The negative after-potential increased by DDT was depressed by catelectrotonus and augmented by anelectrotonus.

10. An attempt was made to interpret the plateau formation in terms of ionic conductances: The plateau is maintained as sodium conductance once raised during the spike is lowered slowly with potassium conductance remaining unchanged. When the slowly declining plateau reaches a certain level, a temporal rise in potassium conductance occurs, which causes a rapid repolarization followed by a positive overshoot.

11. Resemblances were pointed out between cardiac muscle and the DDT-poisoned axons in the shape and mechanism of production of the action potential and in behaviors of the membrane potential.

ACKNOWLEDGMENTS

We are indebted to Dr. S. Hagiwara and Dr. A. M. Shanes for criticisms during the course of this study. Thanks are also due to Miss S. Hamada for technical assistance.

LITERATURE CITED

Bülbring, E., M. Holman and H. Lüllmann 1956 Effects of calcium deficiency on striated muscle of the frog. J. Physiol., *133:* 101–117.

Cranefield, P. F., and B. F. Hoffman 1958a Electrophysiology of single cardiac cells. Physiol. Rev., *38:* 41–76.

——— 1958b Propagated repolarization in heart muscle. J. Gen. Physiol., *41:* 633–649.

Frankenhaeuser, B. 1957 The effect of calcium on the myelinated nerve fibre. J. Physiol., *137:* 245–260.

Frankenhaeuser, B., and L. Widén 1956 Anode break excitation in desheathed frog nerve. Ibid., *131:* 243–247.

Hagiwara, S., and N. Saito 1959 Voltage-current relations in nerve cell membrane of *Onchidium verruculatum*. Ibid., *148:* 161–179.

Hodgkin, A. L., and A. F. Huxley 1952 A quantitative description of membrane current and its application to conduction and excitation in nerve. Ibid., *117:* 500–544.

Hodgkin, A. L., and W. A. H. Rushton 1946 The electrical constants of a crustacean nerve fibre. Proc. Roy. Soc. Lond. (B), *133:* 444–479.

Hoffman, B. F., and E. E. Suckling 1956 Effect of several cations on transmembrane potentials of cardiac muscle. Am. J. Physiol., *186:* 317–324.

Hoffman, B. F., C. Y. Kao and E. E. Suckling 1957 Refractoriness in cardiac muscle. Ibid., *190:* 473–482.

Ichioka, M., and K. Konishi 1957 Anode break excitation in single myelinated nerve fibres. Jap. J. Physiol., *7:* 12–19.

Kao, C. Y., and B. F. Hoffman 1958 Graded and decremental response in heart muscle fibers. Am. J. Physiol., *194:* 187–196.

Koketsu, K., J. A. Cerf and S. Nishi 1959 Effect of quaternary ammonium ions on electrical activity of spinal ganglion cells in frogs. J. Neurophysiol., *22:* 177–194.

Narahashi, T., and T. Yamasaki 1960 Mechanism of increase in negative after-potential by DDT in the giant axons of the cockroach. J. Physiol., in press.

Shanes, A. M. 1958a Electrochemical aspects of physiological and pharmacological action in excitable cells. Part I. The resting cell and its alteration by extrinsic factors. Pharmacol. Rev., *10:* 59–164.

——— 1958b Electrochemical aspects of physiological and pharmacological action in excitable cells. Part II. The action potential and excitation. Ibid., *10:* 165–273.

Tasaki, I. 1956 Initiation and abolition of the action potential of a single node of Ranvier. J. Gen. Physiol., *39:* 377–396.

Tasaki, I., and S. Hagiwara 1957 Demonstration of two stable potential states in the squid giant axon under tetraethylammonium chloride. Ibid., *40:* 859–885.

Tobias, J. M. 1948 Potassium, sodium and water interchange in irritable tissues and haemolymph of an omnivorous insect, *Periplaneta americana*. J. Cell. and Comp. Physiol., *31:* 125–142.

Weidmann, S. 1951 Effect of current flow on the membrane potential of cardiac muscle. J. Physiol., *115:* 227–236.

——— 1955 Effects of calcium ions and local anaesthetics on electrical properties of Purkinje fibres. Ibid., *129:* 568–582.

——— 1957 Elektrophysiologie der Herzmuskelfaser, second ed. Japanese translation by K. Matsuda, Kinhodo, Tokyo.

Yamasaki, T., and T. Narahashi 1959a The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon. J. Insect Physiol., *3:* 146–158.

——— 1959b Electrical properties of the cockroach giant axon. Ibid., *3:* 230–242.

The Olfactory Pigment[1]

RICHARD T. JACKSON
Loyola University, New Orleans, Louisiana

The olfactory area of most animals is easily recognized by its brownish-yellow color. The respiratory epithelium that surrounds it is pink in contrast. The pigment that colors the olfactory tissue has been the subject of much speculation. Since the pigment is only found in close association with the olfactory receptors, it has interested those who are concerned with the basic mechanism of olfaction. The object of this research has been to identify the pigment and observe its physical and chemical properties, its occurrence and distribution.

The first question that should be answered concerns the localization of this pigment. Most histology texts state that the pigment is found in the upper part of the supporting cells which surround the olfactory end organ. Gerebtzoff and Schkapenko ('52) state that the pigment is located in Bowman's glands, in the excretion of Bowman's glands, in the liquid layer that covers the olfactory epithelium and, significantly, in the peripheral part of the olfactory receptors. The pigment was known to be a lipid and the latter authors used histo-chemical evidence for localization. On the basis of this and other evidence, Wright ('56) implicated the pigment in a new theory of olfactory stimulation. The present author feels that before exact localization can be accomplished, and before the pigment is implicated in the process of olfactory stimulation, more should be known about its chemical and physical properties.

Occurrence of olfactory pigment in various species

Examining the nasal epithelial tissue *in toto,* the author has observed a yellow-brown pigmentation, of varying degrees in the dog, cat, rabbit, rat, opossum, sheep, calf, and frog. The degree of pigmentation varies within a species and between species. Scores of dogs, rabbits, and opossums have been examined in the course of this and other studies and the author has noticed a marked variance of degree of pigmentation within a species. Some of this variance is due to the physiological state of the tissue when examined. For example, animals with rhinitis invariably have lighter pigmentation. In fact, the yellow-brown pigmentation is usually absent. Tissue that has been exposed to the air for some length of time is darker brown. If parts of the nasal and frontal bones are removed in an anesthesized animal, and this unnatural dorsal aeration is allowed to continue for about two hours, the exposed olfactory tissue becomes dark brown. Because of these many differences in color within a species, the author doubts that it would be possible to easily catagorize a species' pigmentation.

Pigmentation of the olfactory area is widespread among the vertebrates. In fact, a definite lack of pigmentation has yet to be reported. From this observation, one is led to one of two conclusions. Either the pigment is concerned with the process of olfaction, or it is a direct result of a metabolism peculiar to this tissue and has nothing to do with the basic mechanism of olfaction.

Albinism and anosmia

Various authors quote this relationship. Many of the views concerning this relationship originate with Hermann ('18). Moncrieff, in his book, "The Chemical Senses" ('44), elaborates on Hermann's notions.

The notion is based on the observation that albino animals (e.g., sheep) eat and are poisoned by certain plants that black sheep avoid. The reader is given the impression that albino animals are unable to smell the poisonous plant because of a lack of pigmentation of the olfactory epithelium. Also, one is supposed to be able to

---

[1] Supported in part by the Division of Neurological Diseases and Blindness, N.I.H.

classify an animal as micro- or macrosmatic by the degree of pigmentation of its olfactory epithelium. The present author has examined scores of albino rats and rabbits, judged to be albino because of pink eyes and complete lack of pigment in the fur, and found them to possess a normally pigmented olfactory epithelium. Albino rabbits have been tested electrophysiologically with odors and were found to give data comparable to their pigmented brethren. The division between micro- and macrosmatic seems to rest on observational studies that have not been too exact. Before one could begin to correlate the amount of pigment and the acuity to odor, much more exact data would be needed.

Pigment localization

Gerebtzoff and Schkapenko ('52) were the first to attempt a comprehensive histochemical analysis of the olfactory pigment. These authors used Sudan black, scarlet red, Nile blue sulfate and Feulgen's plasmal reaction. Localization was based mostly on the plasmal reaction. Gerebtzoff concluded that the pigment is formed largely of saturated and unsaturated lipids that are acetals or easily oxidized to acetals. He further concluded that since the pigment is located in Bowman's glands and in the sensory cell itself, it could not be looked upon as a waste product.

The present author considers the above histochemical methods insufficiently precise to permit exact localization. The supporting cells and olfactory receptors are known to contain mitochondria in the same area in which the stained particles were located. Neutral fat, fatty acids, and phospholipids are also found here. Using the above methods, how would one know if only a particular yellowish-brown lipid was being stained?

Heusghem and Gerebtzoff ('53) attempted a chemical isolation of the olfactory pigment. These authors used the combined tissue of 4 dogs, 9 cats, and 11 rabbits. They concluded that the olfactory pigment is essentially constituted of fatty acids. These acids are formed, for the most part, of acetals, or are easily oxidized to acetals. They further concluded that the coloration was due not only to these lipids, but also to non-lipid substances, bound to lipids in the sensory cell and in Bowman's glands. Perusal of the latter data led the present author to believe that the larger part of the pigment was not of a fatty acid character and prompted a reinvestigation of the chemistry of this pigment.

EXPERIMENTAL PROCEDURE

The classical Soxhlet method for fat extraction was used in all but the preliminary testing. Olfactory tissue from a particular animal was weighed, dehydrated *in vacuo* or in absolute alcohol, extracted in a Soxhlet apparatus with absolute alcohol and then ethyl ether. The solutions were filtered, pooled and evaporated to dryness *in vacuo*. Both the alcohol and ether solutions were colored yellow; in most cases, the ether solution had the more intense color. The dried pigment had a fruity odor that usually masked a more basic fishy odor. Many preliminary tests were performed on this dried pigment in an effort to classify it. This crude olfactory pigment exhibits most of the tests of a chromolipid (Fox, '53). The crude pigment was chromatographed using ether and water (95:5). A yellow pigment was easily visible in the moving front. After 4 hours the pigment had an R_f value of 0.90. Two yellow bands were visible on the paper; one band did not move, the other followed the solvent front. Both bands exhibited a bluish-green fluorescence under ultra violet radiation. Various chromatographic color tests suggested the presence of lipids and nitrogen. The olfactory tissue was carefully examined for evidences of fluorescence. Anesthesized dogs and rabbits whose olfactory tissue had previously been exposed, were examined with an ultraviolet lamp by an observer who was completely dark adapted (25 minutes). The tissue did not fluoresce when exposed to common odorants such as amyl acetate, orange, or rosam. None of the extracts of fractions of the olfactory tissue fluoresced until they were applied to chromatographic paper and separated.

The dried olfactory pigment was tested for the presence of phosphorus by the method of Fiske and Subbarow. Phosphorus was present in the ether extract, the alcoholic extract and the pooled dried pigment. The values ranged from 3.86 to 1.9% of the dried samples. Several samples of the crude pigment were tested for nonprotein nitrogen using the Koch and McMeekin procedure. Nitrogen was found in most of the samples. The values ranged from 4.73 to 0% of the dried samples. Because of the behavior of the olfactory pigment toward various organic solvents, its behavior in water and the occurrence of phosphorus and nitrogen in the samples, the author was led to believe that the pigment was a phospholipid. In order to compare the gross characteristics of the olfactory tissue to a known source of phospholipid, the brain and olfactory tissue of a rabbit were subjected to a phosphatide fractionation according to a method outlined by Desnuelle ('52). The brain and olfactory extracts behaved in a similar manner during the separation procedure. Two fractions were yellow in both the brain and olfactory extracts; the phosphatidic acid and the lecithin fraction. These two fractions also exihibted the same chromatographic color tests as the crude olfactory pigment. The absorption spectra of the olfactory pigment fractions showed no peaks between 300 mμ and 800 mμ which is probably an indication of the heterogeneous nature of the extract. The tissue from two cats and two rabbits was carefully processed according to the aforementioned extraction procedure. The results are given in table 1.

Certain of the above extracts were analyzed for phosphorus and nitrogen content. The results are presented in table 2. Of the two yellow fractions; the phosphatidic acid and lecithin fractions, most of the color was found in the former. This relatively crude fraction contained no nitrogen. Approximately 0.6% of this phosphatidic acid fraction obtained from olfactory tissue was phosphorus. An "ideal" phosphatidic acid calculated from the molecular weights of known species contains between 4 and 6% phosphorus. It is easily possible that the accumulation of the pigment in the phosphatidic acid fraction is an artefact produced by oxidation. It seems more likely that the pigment is mainly composed of the more prevalent "lecithines."

TABLE 1

Animal	Wt. of dried extract	Total lipid	Acetone-soluble lipid	Phospholipids
	gm	%	%	%
Cat 1	0.30	7.5	3.85	3.48
Cat 2[1]	0.242	6.6	3.76	2.77
Rabbit 1	0.35	6.0		
Rabbit 2[1]	0.29	5.4		

[1] These tissues were dehydrated *in vacuo*, the tissues of cat 1 and rabbit 1 were dehydrated in absolute alcohol.

TABLE 2

Fraction	P	N	N/P
	%	%	
Cat 1			
Acetone-soluble lipid	0.61		
Phospholipids	1.9		
Cat 2			
Acetone-soluble lipid	0.64		
Phospholipids	2.47		
Rabbit 1 Total lipid	2.12	1.47	0.69
Rabbit 2 Total lipid	2.27	1.58	0.69
Dog 1 Total lipid	3.86	4.73	1.23
Dog 2 Total lipid	2.04		

Lea ('56) has demonstrated that phospholipids become brown in direct relation to the amount of oxygen absorbed. While this absorbed oxygen is being converted to peroxides, the iodine value decreases and free amino groups disappear. The same author suggests that hydroperoxides, epoxides, aldehydes, and other oxidation products of fats are possible participants in such reactions.

It seems evident that browning on exposure to oxygen is a characteristic of phospholipids. The brown pigment that was extracted from the olfactory tissue of various animals has the characteristics of phospholipids. This idea is strengthened by the observation that the olfactory tissue is much darker when it has been exposed to atmospheric air for an hour or so. Air has easy access to the receptor side of the tissue, this could account for the color of the tissue *in situ*. One could then ask why the olfactory tissue is yellow-brown and the respiratory tissue is pink. The most striking anatomical difference is the vast dissimilarity in degree of innervation of the two tissues. There are 50 million olfactory nerve fibers on each side of the nose. While the respiratory tissue is richly innervated it does not approach the above figure. The olfactory nerve is composed of unmyelinated "C" fibers with an average diameter of 0.2 micron (Gasser, '55). The accumulation of yellow lipid droplets in unmyelinated nerve fibers is fairly common. Malhotra ('57), using phase-contrast microscopy, found large lipid spheroids in spinal ganglion cells of adult frogs. This pale yellow material is not found in larval stages, and increases in amount as the animal gets older. Lipid spheroids of a similar nature have been described by Baker ('44) in the anterior mesenteric ganglion cells of the rabbit, by Thomas ('48) in the sympathetic neurones of the rabbits and mouse, and Thomas ('51) in the Purkinje cells of the cerebellum of the rabbit, rat, and mouse. Murray and Stout ('47) described similar inclusions in the adult human sympathetic ganglion cells grown *in vitro*.

Nerve transection studies

It seemed that the easiest way to histochemically identify the olfactory pigment and study its role in the perception of odor, would be to cause either an increase or decrease in its concentration. Under the assumption that the pigment production is actively controlled, an attempt was made to change pigment production by nerve transections. Three rabbits were subjected to unilateral cervical sympathectomy. If the pigment production was controlled through this nervous pathway, the olfactory epithelium on one side of the nose would be a different color than on the other side. The animals were killed 10, 20, and 30 days after the operation. The color of both sides of the nose in the three cases was the same. The same procedure was followed with the ethmoidal nerve, a branch of the opthalmic division of the trigeminal nerve. The ethmoidal nerve has both motor and sensory branches. The sensory branches respond to both tactile and odorous stimulation (Beidler and Tucker, '55). The motor branches contain both sympathetic and parasympathetic fibers. An examination of the olfactory epithelium of animals in which the ethmoidal nerve was cut showed no difference in color. Le Gros Clark ('57) has shown that ablation of the olfactory bulbs causes about one-half of the olfactory receptors to degenerate. Uni- and bilateral ablation of the olfactory bulbs was carried out on several rabbits to ascertain if this degeneration affected the olfactory pigment. The assumption was made that if the pigment was mainly in the olfactory nerve-receptor unit, degeneration of half of these units would produce a visible change in color. These experiments were also negative. Negative results are not as meaningful, but they show that the most obvious treatment of the tissue produces no visible change in the olfactory pigment.

DISCUSSION

The olfactory pigment is composed of phospholipids, probably lecithins, plus the products of auto-oxidation of these phospholipids. The latter undoubtedly contribute most, or all, of the color. Any attempt at histochemical localization will be doubly endangered. First, the auto-oxidation products are unstable and relatively unknown. Second, current histochemical procedures are not sufficiently specific for precise localization.

The accumulation of yellow fat droplets in old, unmyelinated nerve fibers is pointed out by Malhotra. Since the olfactory nerves are unmyelinated and the olfactory tissue is yellow, one may look for a common basis. It is possible that a form of lipid metabolism peculiar to unmyelinated nerves produces the yellow pigment. The yellow fat globules normally extant in an adult nerve could remain in the tissue and continue to contribute to the color even after the nerve degenerated. But the lack of success, in the nerve transection studies points toward the notion that the pigment is a product of non-nervous tissue such as Bowman's glands or the supporting cells.

Contrary to the beliefs of Moncrieff and others, the present author does not believe that the color of the olfactory tissue is an index of the animal's acuity to odors. It has already been mentioned that the color is very similar, if not identical, from species to species. Albino animals have the same degree of color saturation as a pigmented animal of the same species and give the same electrophysiological responses to odors.

If Wright's electron excitation theory is correct, some degree of fluorescence should occur when the olfactory nerves are being stimulated by odors. The absence of fluorescence is a point of disagreement with the theory.

A function of the olfactory pigment that has not been mentioned concerns its possible bactericidal and fungicidal properties. Rothman ('54) points out that the presence of unsaturated faty acids play a role in the disappearance of bacteria from the skin surface. For example, fungal lesions of ringworm and athlete's feet very seldom occur in the presence of functional sebaceous glands. The olfactory tissue is very rich in unsaturated fatty acids and is also in a very vulnerable position in regard to attack by micro-organisms. The observation that the pigment is either reduced or absent in an animal with rhinitis may be evidence for this notion.

SUMMARY

The yellow-brown pigment found in the olfactory tissue is probably due to auto-oxidation products of phospholipids.

The pigment may be a waste product of lipid metabolism peculiar to unmyelinated nerve fibers.

Evidence is given against the supposed relationship between albinism and anosmia.

Some evidence is presented that is in disagreement with Wright's theory of olfactory stimulation.

LITERATURE CITED

Baker, J. R. 1944 The structure and chemical composition of the Golgi element. Quart. J. Micr. Sci., *85:* 1–71.

Beidler, L. M., and D. Tucker 1955 Response of nasal epithelium to odor stimulation. Science, *122:* 76.

Desnuelle, P. 1952 Structure and properties of phosphatides. In: Progress in the Chemistry of Fats and other Lipids, ed. R. T. Holman et al. Academic Press, vol. 1, p. 70.

Fox, D. 1953 Animal Biochromes and Structural Colors. Cambridge Univ. Press, New York.

Gasser, H. S. 1955 Olfactory nerve fibers. J. Gen. Physiol., *39:* 473.

Gerebtzoff, M. A., and G. Schkapenko 1952 Researches sur le pigment de la muqueuse olfactive. C. R. Assoc. Anat., *68:* 511–516.

Herrman, E. 1918 Pilzgeruche. Pharm. Zentr., *59:* 1–4, 7–10, 19–21.

Heusghem, C., and M. A. Gerebtzoff 1953 Resultats concordants des analyses biochemiques et histochimiques des lipides de la muqueuse olfactive. C. R. Soc. Biol., *147:* 540–541.

Lea, C. H. 1956 Some observations on the preparation and properties of phosphatidylethanolamine. In: Biochemical Problems of Lipids, ed. G. Popjak and E. La Breton. New York Interscience Publ. Inc., pp. 81–90.

Le Gros Clark, W. 1957 Inquiries into the anatomical basis of olfactory discrimination. Proc. Royal Soc., B, *146:* 299–319.

Malhotra, S. K. 1957 The cytoplasmic inclusions of the aging neurones of the frog, Rana tigrina. La Cellule, *58:* 365–386.

Moncrieff, R. W. 1944 The chemical senses. J. Wiley & Sons, New York, pp. 3, 118, 216.

Murray, M. R., and A. P. Stout 1947 Adult human sympathetic ganglion cells cultivated *in vitro.* Amer. J. Anat., *80:* 225–273.

Rothman, S. 1954 Physiologyy and Biochemistry of the Skin. Univ. of Chicago Press, p. 318.

Thomas, O. L. 1948 A study of the spheroid system of the sympathetic neurones with special reference to the problem of neurosecretion. Quart. J. Micr. Sci., *89:* 333–350.

——— 1951 A comparative study of the cytology of the nerve cell with reference to the problem of neuro-secretion. J. Comp. Neurol., *95:* 73–102.

Wright, R. H., C. Reid and H. G. V. Evans 1956 Odor and molecular vibration. (3) A new theory of olfactory stimulation. Chem. and Industry, *37:* 973–977.

he Effect of Ribonuclease on the Dialysis of Calcium rom Homogenates of Lobster Nerve and the inding of Sodium in the Homogenates[1]

F. HERMANN RUDENBERG[2] AND JULIAN M. TOBIAS
Department of Physiology, University of Chicago[3]

The following investigation was underaken to answer the question “Is the physcal state of calcium in nerve related to he presence of, and the degree of polyerization of, ribonucleic acid (RNA)?”

To begin with, there is a certain amount f explicit evidence that part of the calium in nerve is bound to something Simon and Szelöczey, ’28; Tipton, ’34; Iehes, Valyi-Nagy and Sokoray, ’35; Solmon and Tobias, ’51; Soloway, Welsh nd Solomon, ’53; Hodgkin and Keynes, 57). In addition, it is known that some f this fraction must be in the form of a et negatively charged complex, since it igrates anodally in an electrical field Mehes et al., ’35; Solomon and Tobias, 51; see also Mazia and Clark, ’36). The ailure of Hodgkin and Keynes (’57) to nd such anodalward migration of labeled a in isolated squid axons suggests either hat (a) the voltages used were insuffiient to move the complex, or that (b) he isotopic Ca did not have time to enter he complex and label it, or that (c) the nodally migrating Ca is extracellular.

Because of the central role of Ca in xcitability phenomena, it then becomes matter of importance to seek out what he bound Ca is bound to. In this conext a number of findings have suggested hat nucleic acid compounds may be inolved. Thus, it has been reported that ixed wavelength ultraviolet light can rease Ca from binding in serum (Clark, 3) or in cells (Mazia and Clark, ’36; shkenaz, ’38), and there is an interacon between ultraviolet radiation and calium which affects rubidium and phoshate uptake by bean root tissue (Tanada, 5). It is interesting to place these reults in juxtaposition with observations f Engström (’43) who correlated an absorption at 2570 A with the ash of microincinerated ganglion cells, and those of von Muralt and Stämpfli (’53) which related an increase in threshold of the frog single fiber preparation to irradiation at 2600 A. In addition, Lansing and Rosenthal (’52), stimulated by experiments of Neuberg and Roberts (’49) on the binding and solubilization of polyvalent cations by nucleotides, felt that in Elodea cells and Arbacia eggs they had demonstrated a relationship between RNA and the binding of Ca or Sr. More recently, Passow et al. (’59) have speculated that some Ca may be bound to nucleic acid complexes in the yeast cell surface.

This report will present evidence that a fraction of calcium is made dialyzable from lobster nerve homogenates by the enzyme ribonuclease (RNAse). It will also be shown that sodium is not dialyzable to the same extent as is potassium, this suggesting that some sodium is bound to a non-dialyzable component of the homogenate.

MATERIALS AND METHODS

Nerves from claws and walking legs were removed from 4 lobsters (Homarus americanus) by the pull-out method, blotted, pooled and weighed. The nerve pool, about three gm, was homogenized in an iced, glass homogenizer for three to 5 minutes and then diluted with 12 ml of glass re-distilled water, this producing a 20% homogenate. Two-milliliter aliquots of this homogenate and 0.5 ml of RNAse (5 mg/ml), or control solution, were subsequently transferred into soaked sacs of dialysis tubing (Visking 12/32″ or 18/32″ sausage casing), resulting in a 16% homogenate. About 2 ml of the diluted homogenate were not dialyzed, and served for control analyses. The sacs were placed in 10 times their volume (25 ml) of glass re-distilled water.

---

[1] This work was done during 1953–54, and a preliminary note was published in Federation Proceedings, *13:* 1954.

[2] Present address: Department of Physiology, The University of Texas Medical Branch, Galveston, Texas.

[3] This work has been supported in part by a grant from the United States Public Health Service and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Six sacs could be dialyzed at one time, with constant stirring, for ¼ to 4 hours at room temperature (24–25°C usually). At the end of an experiment each sac was removed, its content squeezed out into a weighed crucible, and the pH was measured with pH paper (always *circa* pH 6.4). Duplicate weighed aliquots (0.5 ml) were removed from each crucible to be analyzed for RNA and for DNA by the method of Ogur and Rosen ('50). The dialysates were transferred to crucibles but were not analyzed for nucleic acid.

The crucibles were dried to constant weight, heated at 500°C for 24 hours in the presence of 4 N H_2SO_4 to ash the contents, and the ash was then analyzed in triplicate for Ca according to the method of Sendroy ('42, '44), and for K and Na with a Perkin-Elmer flame photometer using Li as the internal standard.

Ribonuclease was purchased as the salt-free crystalline preparation from the Worthington Biochemical Co. The activity of all RNAse preparations was determined according to Kunitz ('46). Protease contamination of the RNAse was tested for by the Anson method ('38). When compared to trypsin the contamination, if any, was less than 0.01% by weight. In order to make more certain that there was no protetase activity, some dialyses were done with RNAse that had been boiled for 5 minutes in 0.01 M acid, cooled and neutralized to phenol red with dilute base. The RNAse activity of these preparations

TABLE 1

Calcium egress from lobster nerve homogenates treated with RNAse in distilled water

Tubing size	Dialysis duration	Experiment number	Per cent of total Ca dialyzed out	
			Control	Active RNAse
inches	*hours*			
12/32	2	8	32.9	40.6
		12	44.4	48.4
	3	5	41.5	57.1
		8	41.0	52.0
		9	50.5	62.8
		10	47.5	57.4
		10	47.0	59.4
		12	50.0	51.4
		13	48.6	55.5
	4	8	50.3	58.9
		11	55.5	63.0
		11	53.5	60.0
18/32	¼	17	12.5	15.5
	½	16	19.4	27.9
		17	—	29.5
		18	21.0	38.4
	1	15	44.5	57.3
		16	35.4	—
		19	34.3	53.7
	2	15	57.9	63.6
	3	13	66.0	69.0
		14	64.3	75.4

was either identical with or only slightly ess than that of the unboiled fresh enzyme. Some experiments were done with RNAse that had been completely inactivated by boiling for 60 minutes in 0.01 M base, cooled and neutralized to phenol red with dilute acid. There was no RNAse activity detectable in preparations treated in this way.

RESULTS

To make maximum use of the lobster tissue, and because about 40 mg of wet tissue were needed for each salt or nucleic acid analysis, each dialysis sac contained about 400 mg of tissue equivalent and was dialyzed until about 50% of the calcium had permeated. Later, it appeared that shorter dialysis might show up a greater difference in Ca egress between sacs with or without added RNAse. The data are shown in table 1. It may be seen that in every case the presence of active RNAse promoted the egress of Ca from the dialysis sac. The data are also presented in figure 1. RNAse increased the dialysis by an average of about 9%.

The RNAse preparation which was rendered completely protease free by heating had a slightly smaller effect on the Ca dialysis than did the unheated RNAse, as would be expected if the heating also decreased the RNAse activity somewhat. Even so, the Ca egress was still 6.3% greater on the average than in the controls in 5 of the 6 experiments done in this way. Complete inactivation of the RNAse eliminated its effect on the dialysis rate entirely.

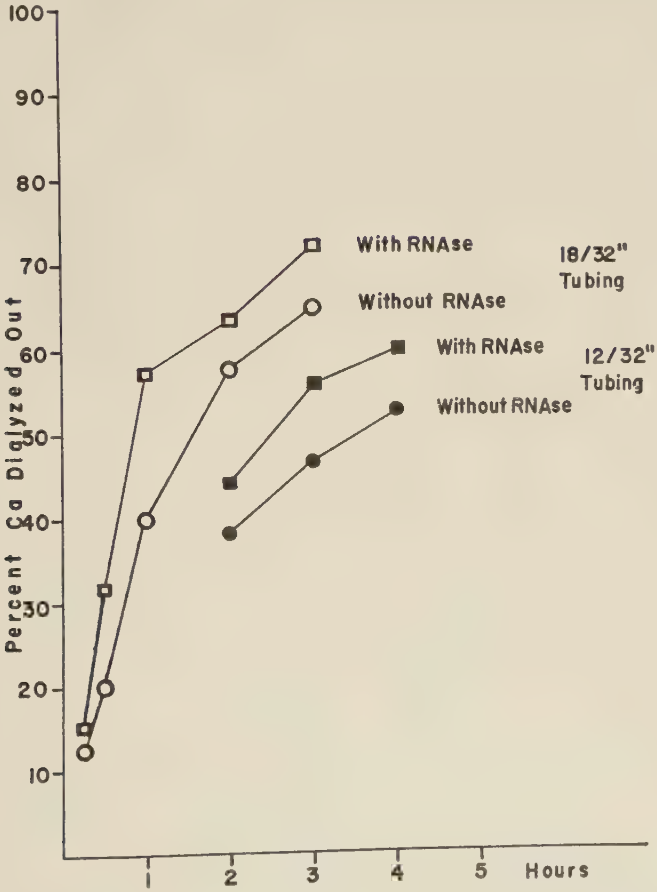


Fig. 1 The effect of RNAse on the egress of Ca from dialysis sacs containing lobster nerve homogenates in distilled water.

TABLE 2

Potassium egress from lobster nerve homogenates treated with RNAse in distilled water

Tubing size	Dialysis duration	Per cent of total K dialyzed out[1]	
		Control	Active RNAse
inches	*hours*		
12/32	2	81.5±1.5 (2)[2]	83.2±2.2 (2)
	3	84.6±0.8 (7)	86.1±0.8 (7)
	4	85.9±0.5 (3)	86.3±0.2 (3)
18/32	¼	62.1 (1)	66.0 (1)
	½	74.3±1.9 (3)	78.0±1.7 (3)
	1	83.6±1.2 (2)	89.0 (1)
	2	88.2 (1)	88.6 (1)
	3	87.7±2.6 (2)	88.0±0.0 (2)

[1] Data given as ±S.D.

[2] Number of items shown in parentheses.

TABLE 3

Sodium egress from lobster nerve homogenates treated with RNAse in distilled water

Tubing size	Dialysis duration	Per cent of total Na dialyzed out[1]	
		Control	Active RNAse
inches	*hours*		
12/32	2	76.0±2.5 (2)[2]	77.1±1.5 (2)
	3	80.0±0.6 (7)	81.0±0.8 (7)
	4	80.1±0.8 (3)	81.1±0.4 (3)
18/32	¼	50.4 (1)	53.5 (1)
	½	65.5±3.0 (3)	67.5±1.8 (3)
	1	76.5±1.5 (2)	80.0 (1)
	2	81.1 (1)	82.1 (1)
	3	82.2±0.4 (2)	82.7±0.2 (2)

[1] Data given as ±S.D.

[2] Number of items shown in parentheses.

TABLE 4

Egress of organic matter, in terms of dry weight, from lobster nerve homogenates treated with RNAse in distilled water

Tubing size	Dialysis duration	Per cent of dry weight dialyzed out[1]	
		Control	Active RNAse
inches	*hours*		
12/32	2	40.9±0.6 (2)[2]	42.3±1.0 (2)
	3	43.0±1.0 (7)	45.4±1.2 (7)
	4	44.1±0.2 (3)	45.2±1.6 (3)
18/32	¼	24.4 (1)	27.7 (1)
	½	32.8±2.9 (3)	34.6±2.5 (3)
	1	40.2±0.5 (3)	40.0±2.0 (2)
	2	41.5 (1)	44.6 (1)
	3	44.8±0.3 (2)	46.6±1.2 (2)

[1] Data given as ±S.D.

[2] Number of items shown in parentheses.

While Ca dialysis rate increased when NAse was added, there was a probably egligible effect on the rate of dalysis of K, Na or other diffusible solids as interpreted in terms of dry weight. Tables 2, 3 and 4 isplay data for the mean per cent dialysis f K, Na and dry weight. There is no staistically significant difference between olumns in these three tables, and thereore no effect of RNAse is apparent. Howver, in all cases the averages for ion egess were very slightly higher when RNAse vas used. Conceivably a better analytical nethod could show a real effect. All of he data are plotted in figure 2 in terms of per cent dialyzed out as a function of time, ndependent of the RNAse. The asympote toward which the Na egress curve approximates is somewhat lower than that vhich the K egress curve approaches.

Figure 3 expresses the data for polymerized RNA remaining in the dialysis sac. The differential solubility method of Ogur and Rosen determines only polymerized nucleic acids. The depolymerized RNA most probably dialyses out. Dialysed nucleotides could not be determined because the presence of non-nucleic acid nucleotides interferred with their estimation. The RNA analyses were done primarily to establish whether or not the RNAse was really active in the presence of the nerve homogenate, and it is seen that it was. More nerve material in each analysis would be needed were a greater accuracy desired. Thus, the reversal in slope occurring after one hour in figure 3 cannot now be taken seriously. The apparent breakdown in the RNA that occurs in the absence of added RNAse may result from the action of a

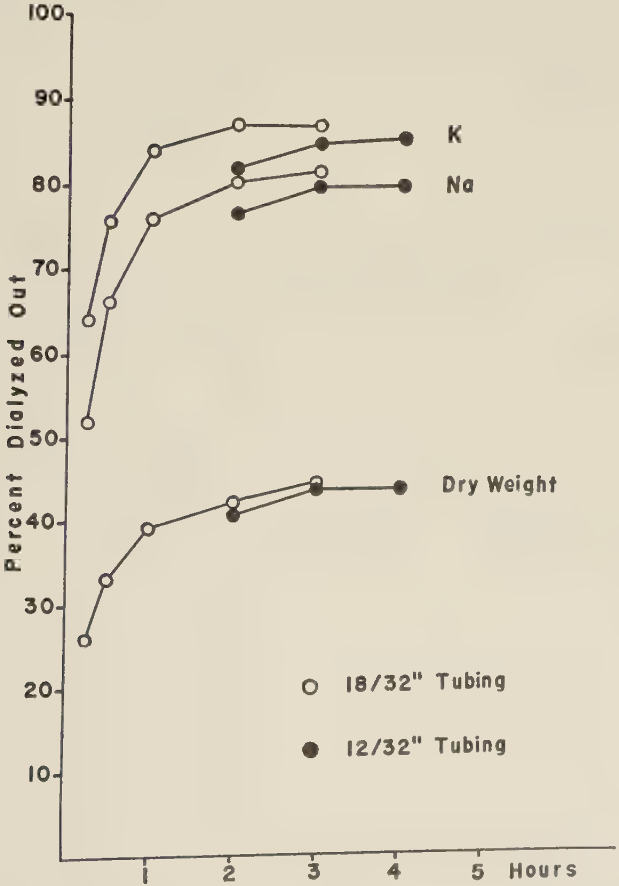


Fig. 2 The dialysis egress of K, Na and diffusible matter (dry weight). RNAse has no significant effect.

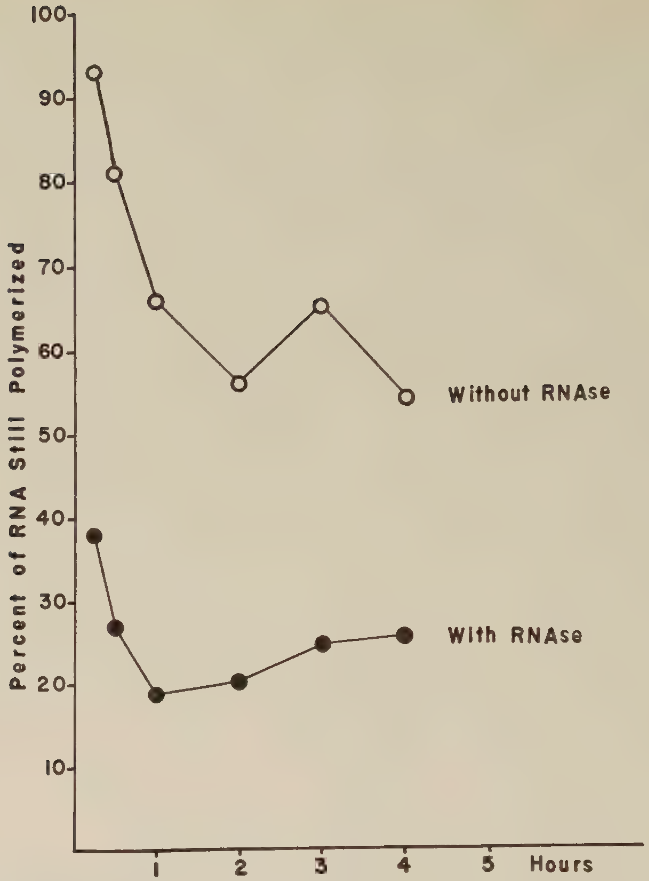


Fig. 3 The effect of RNAse on the polymerized RNA. The depolymerized RNA is presumably free to dialyze.

phosphatase or diesterase, may result from endogenous RNAse activity, or may be an expression of a lower molecular weight fraction of RNA diffusing out which is normally present in addition to the more highly polymerized portion.

DISCUSSION

A comparison of figures 1 and 2 shows that the dialysis egress of K, Na and other solids reaches a plateau, indicating minimal further egress, considerably before Ca egress does. These curves strongly suggest that most or all of the Ca is not as free to diffuse as are the K and Na. Although it is not known whether the Ca that dialyses out does so in the form of Ca ion or as a small complex, it is evident from the dry weight curve that about 55% of the nerve homogenate is still retained at three to 4 hours and could serve as a substrate for the binding of Ca.

Consideration of the volumes inside and outside the dialysis sacs indicates that at equilibrium the ion concentration distribution would show 91% dialysed out if all the ions were freely diffusible. Since only 82.1 ± 0.3% of the Na was dialysed out at equilibrium, whereas some 87.5 ± 0.7% of the K had been removed, therefore a small amount of the total Na seems to have been restrained more firmly than the K. Both Tobias ('50) and Steinbach ('50) have shown that frog muscle binds a similar small percentage of the total Na. The binding agent, which has not been identified, might conceivably be involved in transsurface transport of Na.

TABLE 5

Calcium egress from lobster nerve homogenates treated with RNAse in presence of added salts

Tubing size	Dialysis duration	Solution composition	Per cent Ca out	
			Control	RNAse
inches	*hours*			
12/32	2.5	0.2 M KCl	69.0	75.9
	3.25	0.2 M KCl	76.0	82.7
18/32	1.0	0.2 M KCl	46.2	54.7
	1.0	0.2 M NaCl	56.4	58.6

Because of difficulty in analyzing the dialysate in the presence of large amounts of salt, all the above data have been derived from the Ca remaining within the sac in comparison with an undialyzed aliquot.

Two experiments on the effect of RNAse were carried out in the presence of salt added both inside and outside the sac (table 5). Analysis for small amounts of Ca in the presence of large amounts of NaCl or KCl was found difficult and therefore only the inside compartment or an undialyzed aliquot could be analyzed satisfactorily. The presence of either 0.2 M NaCl or KCl accelerates the egress of Ca as expected. The increase in dialysis due to the presence of RNAse was observed to be about 7% in the presence of KCL and about 2% in the presnce of NaCl, as may be seen in the table. These experiments support the conclusion that the increase in dialysis of Ca produced by the RNAse is not merely a passive redistribution of Ca resulting from a change in the concentration of some other charged ion.

It was not possible to obtain a good correlation between the increased Ca dialysis and the amount of RNA depolymerized. It is possible, however, to calculate for each experiment the maximum amount of Ca that could be bound by the total RNA present and to compare these data with the RNAse-produced increase in Ca dialysis. Assuming that the calcium is bound only to the phosphorous of RNA, it is apparent that the maximum of binding sites can be determined. RNA is known to consist of about 9.5% P, and each 4 P atoms maximally have 4 sites at which Ca could bind (Davidson, '57). Thus, about 0.9 meq. of Ca could be bound by each 100 gm dry weight of nerve, or an average of 8.5 ± 0.3% of the total Ca in each dialysis. The increased Ca dialysis observed was 8.7 ± 0.9% of the total Ca (the datum for the quarter hour dialysis was excluded because of inadequate time for the released Ca to dialyse). The difference between these means is not significant and thus

TABLE 6

Lobster nerve analyses

Item	No. of experiments	No. of analyses	Datum ± S.D.	Range
Total number lobsters	16			
Water content as per cent of wet weight	16	16	87.7 ±0.2	86.3 –88.6
Ash weight as per cent of wet weight	14	14	3.37±0.11	2.88– 4.24
meq. Ca/100 gm wet weight	15	55	1.44±0.04	1.08– 2.08
meq. K/100 gm wet weight	12	44	16.4 ±0.3	11.0 –19.6
meq. Na/100 gm wet weight	12	44	15.5 ±0.3	11.2 –20.0
mg RNA/100 gm wet weight	10	20	28.8 ±0.9	23.4 –37.4
mg DNA/100 gm wet weight	9	18	50.0 ±1.1	41.0 –57.4

Data were collected from experiments in which all limb nerves from several lobsters were homogenized. Untreated aliquots of the homogenates were analyzed in triplicate or quadriplicate for Ca, K and Na, and in duplicate for RNA and DNA (August, 1953 to March, 1954).

indicates that the increased Ca dialysis observed in the presence of RNAse could be wholly accounted for by Ca that might have been bound to RNA.

Actually, about 20% of the total RNA was not depolymerized by the RNAse (fig. 3). The method of Ogur and Rosen ('50) detects depolymerization of nucleic acids by means of differential solubility according to the degree of depolymerization. It gives no information regarding the dialyzability of the depolymerization products, nor regarding the ability of these products to bind Ca. If, however, a lack of depolymerization measured by this method may be thought to imply a lack of Ca release by this fraction of the RNA, then the mean for maximum Ca binding (and releasable by RNAse) should be reduced by 20% of 8.5% or to 6.8%. The difference of the means is however still not significant.

While the most probable mechanism for the increased dialysis of Ca as a result of RNAse action is an effect of the enzyme upon the RNA molecule, there are possible alternatives: (1) The RNAse itself simply displaces Ca from the homogenate. At the pH of these experiments RNAse is positively charged, and it may be calculated by analogy with the charge to molecular weight ratio of albumin, that 10^{-6} moles of charge are added (Klotz, '54). The increase in Ca dialyzed out also happens to be 10^{-6} equivalents maximally. (2) RNAse acts on the nucleoprotein so as to depolymerize the RNA, and because of such structural changes somehow releases Ca from protein rather than from the RNA itself. (3) The polymerized RNA serves as a carrier for any Ca present within the dialysis sac. It is not possible rigorously to decide among these alternatives from the data at hand, since the evidence shows only that RNAse is capable somehow of affecting the ability of Ca to diffuse in this experimental situation.

What does seem clear is that although this system may play subtle, and conceivably important, roles in excitation and other phenomena, no very large amount of Ca, Na or K is firmly bound to RNA. An alternative for Ca localization has been suggested elsewhere (Tobias, '58).

SUMMARY

1. Diluted lobster nerve homogenates were dialyzed against distilled water for various periods of time. In some of the dialyses RNAse was added to the homogenate. The presence of this RNAse increased the Ca dialysis by about 9%. It has not been established whether the Ca dialyses as Ca ion or while bound to some nucleotide.

2. This RNAse-sensitive Ca fraction is independent in magnitude of the large osmotic disequilibrium at the start of an experiment, since it appears also when the egress of K, Na and other diffusible solids have reached equilibrium, and it appears in the presence of added salt. Only questionable effects, if any, of RNAse on K or Na egress have been seen.

3. The greater amount of K than of Na that dialyses out indicates that a few per cent of the Na are still bound to nondialyzable constituents of nerve after three to 4 hours of dialysis.

4. Analytical data for lobster nerve Ca, K, Na, RNA and DNA are presented.

LITERATURE CITED

Anson, M. L. 1938 The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol., *22:* 79–89.

Ashkenaz, E. W. 1938 The release of calcium from muscle on stimulation by ultraviolet radiation. J. Cell. and Comp. Physiol., *12:* 139–147.

Clark, J. H. 1923 The effect of ultraviolet light on the condition of calcium in the blood. Am. J. Hyg., *3:* 481–482.

Davidson, J. N. 1957 The Biochemistry of the Nucleic Acids, 3rd ed. Wiley, New York.

Engström, A. 1943 Korrelation zwischen Aschengehalt und Ultra-violett Absorption bei verschiedenen Zellbestandteilen. Chromosoma, *2:* 459–472.

Hodgkin, A. L., and R. D. Keynes 1957 Movements of labelled calcium in squid giant axons. J. Physiol., *138:* 253–281.

Klotz, I. M. 1954 Personal communication.

Kunitz, M. 1946 A spectrophotomertic method for the measurement of ribonuclease activity. J. Biol. Chem., *164:* 563–568.

Lansing, A. I., and T. B. Rosenthal 1952 The relation between ribonucleic acid and ionic transport across the cell surface. J. Cell. and Comp. Physiol., *40:* 337–346.

Mazia, D., and J. M. Clark 1936 Free calcium in the action of stimulating agents on *Elodea* cells. Biol. Bull., *71:* 306–323.

Mehes, J., T. Valyi-Nagy and L. Sokoray 1935 Der Ca-Gehalt der peripheren Nerven des Frosches und die Verschiebung der Ca-Ionen bei Polarization desselben. Arb. Ungar. Biol. Forschungsinst., *8:* 318–326.

von Muralt, A., and R. Stämpfli 1953 Die photochemische Wirkung von Ultraviolettlicht auf den erregten Ranvierschen Knoten der einzelnen Nervenfaser. Beiträge zur Methode der stroboskopischen Photochemie. Helv. Physiol. et Pharmacol. Acta, *11:* 182–193.

Neuberg, C., and I. S. Roberts 1949 Remarkable properties of nucleic acids and nucleotides. Arch. Biochem., *20:* 185–210.

Ogur, M., and G. Rosen 1950 The nucleic acids of plant tissues. I. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Ibid., *25:* 262–276.

Passow, H., A. Rothstein and B. Loewenstein 1959 An all-or-none response in the release of potassium by yeast cells treated with methylene blue and other basic redox dyes. J. Gen. Physiol., *43:* 97–107.

Sendroy, J. 1942 Photoelectric determination of oxalic acid and calcium, and its application to micro- and ultramicroanalysis of serum. J. Biol. Chem., *144:* 243–258.

——— 1944 Determination of serum calcium by precipitation with oxalate. A comparative study of factors affecting the results of several procedures. Ibid., *152:* 539–556.

Simon, A., and J. Szelöczey 1928 Untersuchungen über Kalium- und Calciumgehalt der peripheren Nervenfasern. Biochem. Zeit., *193:* 393–399.

Solomon, S., and J. M. Tobias 1951 Influence of direct current on the long axis distribution of K, Ca and Na in dog nerve. Biol. Bull., *101:* 198.

Soloway, S., J. H. Welsh and A. K. Solomon 1953 Studies on Ca^{45} transport in crayfish nerves. J. Cell. and Comp. Physiol., *42:* 471–485.

Steinbach, H. B. 1950 Ion binding in muscle homogenates. Am. J. Physiol., *163:* 236–246.

Tanada, T. 1955 Effects of ultraviolet radiation and calcium and their interaction on salt absorption by excised mung bean roots. Plant Physiol., *30:* 221–225.

Tipton, S. R. 1934 The calcium content of frog nerve. Am. J. Physiol., *109:* 457–466.

Tobias, J. M. 1950 Injury and membrane potentials in frog muscle after depleting potassium and producing other changes by soaking in potassium free salt solution or distilled water. J. Cell. and Comp. Physiol., *36:* 1–13.

——— 1958 Experimentally altered structure related to function in the lobster axon with an extrapolation to molecular mechanisms in excitation. Ibid., *52:* 89–126.

hixotropy of Axoplasm and Effect of Activity on Light merging from an Internally Lighted Giant Axon[1]

S. SOLOMON[2] AND JULIAN M. TOBIAS
Department of Physiology, University of Chicago[3] and the Marine Biological Laboratory, Woods Hole, Massachusetts.

There has been much speculation conerning the structural physical properties f axons and the changes which they may ndergo during excitation, conduction and ecovery. (For complete literature citaions on this point and others not explicitly iven in this report see reviews of Tobias, 51, '52, '58; Liudkovskaia, '57; Frank, '58; obias and Nelson, '59.) The probability hat reversible sol-gel interconversions are ssential parts of functioning has been rgued (Heilbrunn, '43), and is supported y observations which suggest that签erence, as by hydrostatic pressure, with metastable colloidal state prevents nornal functioning (Grundfest, '36; Spyropolos, '57, '57a). There is, however, rather ittle information of a direct sort bearing n the dynamic mechanical characteristics f axons. If a point of view such as that f Sutherland ('05, '08), which was rexamined by Fry and Fry ('50) (see also obias reviews and Tobias, '60), which onsiders a possible mechanical componnt of the nerve impulse is to be entertained, it would be valuable to know more about the dynamic characteristics of axoplasm structure.

One reason for doing the following experiments therefore has been to allow visualization of changes in axoplasmic consistency occurring as a result of rapid or slow mechanical stirring and compression with an impaling quartz microneedle.

There has, however, been a second motive for the experiments to be described. It has been reported from a number of sources that certain complex changes in the optical and mechanical properties of nerve occur as a concomitant of activity. The techniques used in the optical experiments have measured changes in the scattering of light incident on the outer surface of the nerve. Since, however, much of the light scattered by such a system is scattered by connective tissue elements and by axonal sheaths, there is a large background of illumination falling on the detector photocell which is scattered there by structures possibly playing little if any direct part in conduction of the propagated disturbance. Nor is it clear from the experiments reported to date that much of the apparent change in light scattering with activity may not in part be due to changes in surface reflectivity, index of refraction, etc., etc. As a first step toward sorting out these possibile parameters of the scattering changes, to make the axoplasmic and surface changes ultimately resolvable, and possibly to introduce a more generally useful analytical tool, we have tried putting a light source, in the form of a quartz microneedle into the axoplasm and have measured photoelectrically the light emerging radially through the axon surface. Since such emergent light also passes through extraaxoplasmic elements this experiment is no more able than the others to resolve intra-from extracellular elements in responsibility for the scattering change. The next step, not yet tried, would be to put a second quartz rod into the axoplasm from the other end of the axon to act as a light receiver and thus possibly to approximate an intracellular spectrophotometer or nephelometer. In this way any part played by the axoplasm alone could be isolated.

---

[1] This work was done in 1951, preliminary note appearing in Biol. Bull., *101:* 213, 1951.

[2] Present address: Department of Physiology, Medical College of Virginia, Richmond, Virginia.

[3] This work has been supported in part by a grant from the United States Public Health Service, and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

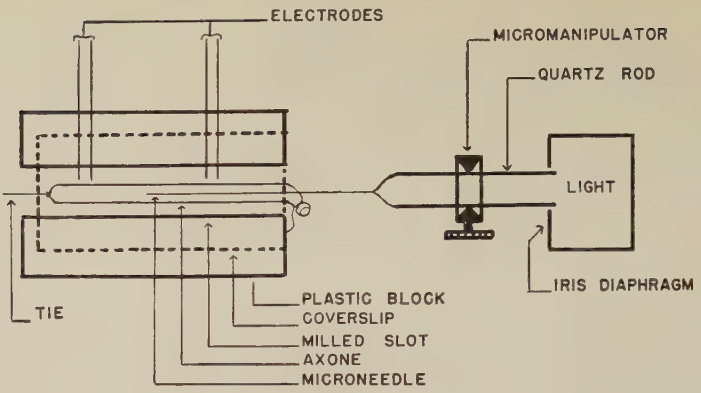


Fig. 1 Diagram of experimental arrangement. Top view, not to scale.

The experiments reported here give direct evidence of the thixotropic nature of squid axoplasm, and also show that the light emerging from an internally lighted axon can be modified somewhere along its path by stimulation and impulse conduction.

METHODS

The squid giant axon, excised and cleaned in filtered sea water, was mounted in a slot (about 0.5 × 0.5 mm) milled in a block of Plexiglas (fig. 1). The block was cut to fit in the mechanical stage of a microscope. Cradled in the slot on appropriate electrodes, and held by silk ties anchored to the block, one end of the axon was draped over an end of the block as shown in the figure. Once the axon was mounted the slot was covered with a cover glass, thus forming a moist chamber. A 5 mm quartz rod, held in a micromanipulator and previously pulled out for several centimeters to an 80–100 μ diameter needle, was then brought into position alongside the microscope holding the block, and the axon, where it curved over the edge of the block, was stabbed by the needle tip. The latter was then advanced for a short distance into the axon. Since stimulating electrodes and monitoring pick-up electrodes were somewhat upstream from this point there is no way of knowing with absolute certainty that the impulses in all experiments actually traversed the region of impalement.

Such a quartz microneedle may be variously used. It is convenient for mechanical manipulation of the axoplasm while the latter is observed microscopically, or it may be used as a conduit to pipe light directly into the axoplasm. In the latter case a regulated light source illuminates the protruding end of the rod (fig. 1). Though it has not been so used to date it could also be made in the form of a capillary micropipette for simultaneous microinjection. For quantitative measurement of changes in light emerging from the impaled axon during activity, when the only light in the field was that brought into the axon via the quartz tip, the block was

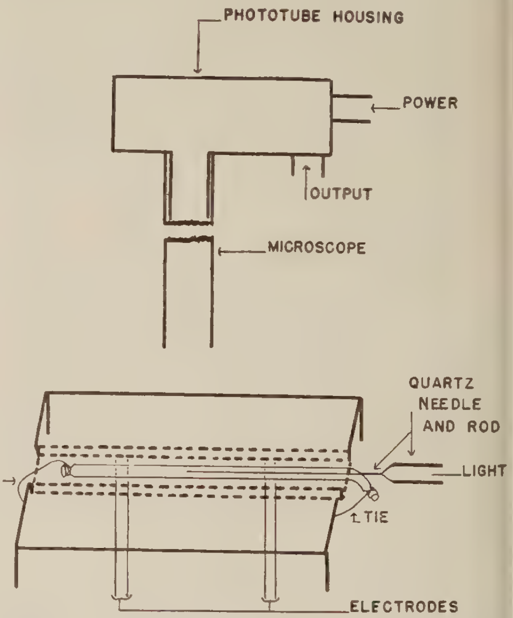


Fig. 2 Diagram of arrangement for measurement of emergent light.

painted completely flat black, and, after visual positioning of the axon and the impaling rod, the microscope ocular was replaced by a photomultiplier tube (RCA no. 931) in a fitted aluminum housing (fig. 2). The output of the photocell was measured with an electronic microammeter (max. sens. 0.01 μamp per 100 div. full scale). Stimulation was by means of a standard stimulator, impulse conduction being monitored in orthodox fashion.

RESULTS

Thixotropy of axoplasm

The squid giant axon observed by ordinary microscopy appears to be optically empty; it is, however, birefringent (Bear, Schmitt and Young, '37; Shaw and Tobias, '51), and therefore must contain oriented, anisotropic particles. Examination of axoplasm in the surviving, excitable lobster axon, by slit ultramicroscopy, shows the presence of various structural elements and regions of different rigidity or viscosity (Tobias and Bryant, '55). Now then, it is also found that if one gently introduces an impaling quartz rod into the squid axon as described above and uses this rod to conduct light into the axoplasm then one sees a bright Tyndall cone extending into the axoplasm ahead of the quartz tip, i.e., the axoplasm is turbid.

If the impaling quartz rod be moved about in the axoplasm characteristic local changes occur. These changes are, however, different depending on whether the axoplasm be vigorously stirred by oscillating the needle back and forth along the long axis or be gently compressed by slowly moving the needle forward.

Relatively vigorous back and forth stirring produces a track in which the axoplasm becomes clearer as the Tyndall beam disappears there, and in which there appear larger particles in Brownian motion. One sees, therefore, what one would expect from breaking a gel. Such a solated area is usually sharply delimited, the axoplasm continuing in its resting condition just beyond. If the system then be left undisturbed the track gradually becomes less prominent and may more or less disappear, the axoplasm tending to resume its usual appearance. Such reversibility is, however, seen only during the early phases of an experiment; after sufficient trials the system remains solated (see also Tobias, '60). When reversal is seen it requires some 10 minutes or so for completion. Such behavior is typical of thixotropic systems (Freundlich, '35; Green and Weltmann, '46). Gross solation of axoplasm after prolonged soaking of the axon in sea water or after exposure to proteases, and its lack of effect on excitability is discussed elsewhere (Tobias, '60).

In contrast to such changes following rapid stirring, quite different results are seen as a consequence of gently compressing the axoplasm ahead of the impaling rod by advancing it slowly and evenly along the axonal long axis. Now there appears in the Tyndall beam ahead of the needle a series of alternate, stationary, light and dark curved bands which are concave toward the rod tip. Cup-shaped about the tip, these bands can be shown, by focussing in depth, to extend to the periphery of the axoplasm around the entire axonal circumference, but not into the extraaxonal sheath. The size, contour and positioning of the bands are, as has been made clear by measurements, a function of the size and shape of the quartz tip. Slow compression of this sort produces no visible evidence of solation, and its consequences indicate an appreciable degree of deformability without breaking on the part of the axoplasmic gel. No particles are seen in Brownian motion as after vigorous stirring.

Before discussing certain implications of these findings a further observation will be recorded.

Effect of activity on light emerging from an internally lighted axon

If one uses the impaling quartz tip as the sole source of light in the field and measures the light emergent from the axon (fig. 2) it is found that stimulation can produce changes in the emergent light. To date only white light or blue light has been used.

Thirteen giant axons have been studied, and changes have been sought during 85 periods of stimulation of these axons. Stimulation has been at a rate of 175 per sec., a voltage of 0.25–0.74, a pulse width of about 0.2 msec., and has lasted for from 80–150 seconds. In these 85 trials the emergent light decreased in 61 (72%), remained unchanged in 23 (27%) and increased in one. In all of these cases the axons were excitable.

The change has always been a small one. Readings were made with a meter sensitivity of 0.1 μamp per 100 divisions full scale, and since the changes have been of the order of 2–5 μamp this means that they actually were displacements of only 2–5%. A few experiments would therefore not have been taken very seriously. However, the facts that (a) such deflections occurred in 72% of the cases when stimulation was applied, that (b) among these responses 94% showed recovery (87% convincingly, 7% questionably, 6% no recovery), and that (c) similar deflections during 87 rest periods before or between stimulations were seen in less than 6% of the cases contributes largely to credibility. The sort of random fluctuations encountered is illustrated in figure 5 which also shows an isolated response to 120 seconds of stimulation.

That the change when it occurs depends upon impulse propagation is argued, as in the case of changes seen with extracellular light sources, by the facts that (a) 17 trials with just subthreshold stimulation failed to produce the change in emergent light, (b) in 9 out of 10 trials the emergent light returned to the resting level after impulse conduction stopped even though stimulation at a subthreshold level was continued, and (c) supramaximal stimulation failed to produce any optical change in 6 nerves (12 trials) which for various reasons had ceased to conduct.

In figure 3 there is plotted an average curve which shows the change in emergent light in all axons showing a change, as a function of stimulation and time. This curve is simply the average of a scatter graph and no distinction was made among axons stimulated for different times. Figure 4, however, shows average curves describing both response and recovery, the curves being grouped according to stimulation duration. Figure 5 shows an isolated response and recovery curve for an individual axon stimulated for 120 seconds.

It may be noted that blank spaces have been left in the curves for some seconds following the start and cessation of stimulation. This has been done deliberately so as to avoid the implication that the precise temporal relation of the optical change to the electrical change (spike) is known. In most cases there was no clear-cut deflection of the meter for some 2–20 seconds after the start of stimulation. So great a delay could not be due to meter lag, the meter requiring only a fraction of a second for response. Even so, however, a rapid onset of the change could have been missed if it were at first so small

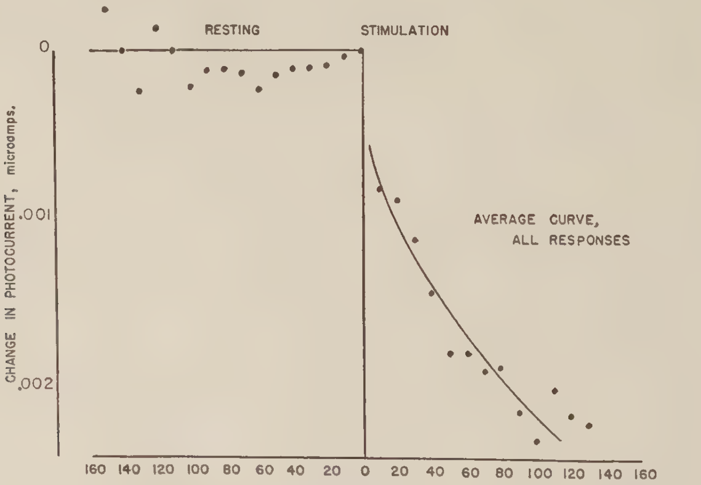


Figure 3

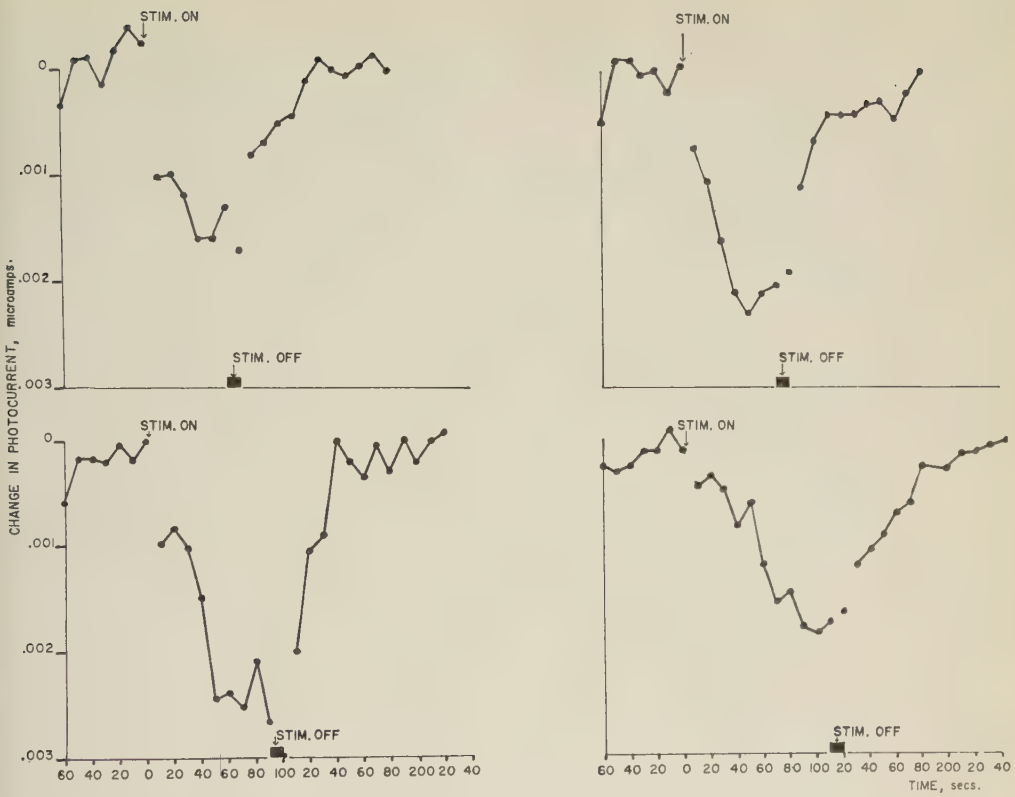


Fig. 4 Average responses to different stimulation times.

as to be beyond the sensitivity of the measuring system. For these reasons it is felt that the shape of the curves is not well enough established to warrant temporal correlations. Whatever the precise time characteristics of the optical response and recovery curves, it is clear that the change well outlasts the stimulation period producing it.

With this technique there has at no time been seen any increase in the emergent light preceding the decrease. It will be recalled that scattering of externally incident light by whole nerve may show monophasic or diphasic changes with activity, and that these changes depend for their direction largely on the tension applied to the nerve (Bryant and Tobias, '55). This stretch effect not being known when the present work was done, comparable experiments were not done with the quartz rod impaled axon.

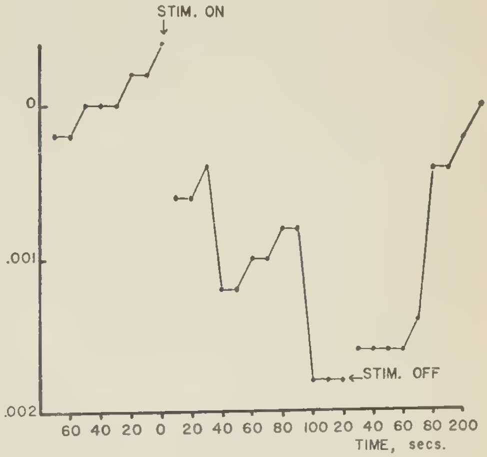


Fig. 5 Individual response.

DISCUSSION

The theory of thixotropy is still incomplete (Verwey and Overbeek, '48). Several points, however, seem to meet with more or less general agreement; (a) "the thixotropic state can best (or perhaps only) be obtained with sols containing disc-shaped or rod-shaped particles." Axoplasm is such a sol as is revealed by birefringence studies. (b) A particular micellar orientation is probably necessary for the thixotropic gel state to exist (oriented coagulation) (Glasstone, '46), and (c) the zeta potential of the particles making up a thixotropic sol will be in some narrow range between the high value required for stability and the low one leading to coagulation. In addition, whatever be the correct picture of gel formation, it seems likely that in dilute thixotropic systems the gel will be a loose network of particles, interconnected at only a few points, in which the dispersion medium is immobilized, collapse of the whole being prevented by repulsive forces between other parts of the particles.

It appears from these experiments that squid axoplasm is a thixotropic system. At least two other inferences, however, seem warranted. The facts that a microneedle (80–100 μ diameter) advanced slowly along the interior of axons 4–5 times that size need not produce breakdown of axoplasmic structure, and that, on the contrary, such an advancing rod is met by an apparent plastic, yielding resistance on the part of the axoplasm, resulting in the appearance of a non-solated material exhibiting cup-shaped banding, strongly imply that the axoplasmic gel is made up of a framework whose elements are probably rather strongly cross bonded. This is consistent with the observation that squid axoplasm is highly elastic (Tobias, '60). These inferences are supported by electron microscopic studies which show more or less randomly arranged threads of material traversing the axoplasmic cross section. In life these fibrils probably have a preferential orientation parallel to the axon long axis (Tobias and Bryant, '55).

It might also be expected that such a system would be rheopectic. By rheopexy (Freundlich and Juliusburger, '35; Juliusburger and Pirquet, '36) is meant the "solidification of thixotropic sols by gentle and regular movement." Thus, if a sol of V_2O_5 be made thixotropic with H_2SO_4, solidification may require some 60 minutes of standing after solation is induced by shaking. If, however, the tube containing the sample be then gently rolled to and fro between the palms of the hands the V_2O_5 may gel in some 15 seconds and may do so with the formation of annular striations. Photographs suggesting similar changes with cross banding in certain soap solutions as a result of capillary flow have been published by Philippoff ('38).

Characterization of the axoplasm as rheopectic also gives more meaning to an observation of Flaig ('47) who, working with the squid giant axon, found that gently rolling the axon between the fingers for a few seconds produced an increase in turgor or stiffness which rapidly subsided when such finger rolling was stopped.

It is possible that the observations reported here might be ascribed to dilatancy (Reynolds, '35). Repetitive banding, however, is not characteristic of dilatancy, and dilatancy occurs in systems rich in particles and relatively poor in water. Axoplasm is not such a colloid.

The fact that the axoplasm is thixotropic is consistent with the notion of delicate poise subserving ease of interconversion between the gel and sol states, there being at the same time an ever present tendency to reconstitute the organized system. The property of rheopexy may allow the system to be exposed to certain kinds of mechanical disturbance (perhaps those of respiratory movements, rhythmical smooth muscle movements, cardiac movements) without necessarily undergoing any large change in state. This, of course, is an extrapolation, and one does not know now whether such movements are not quantitatively great enough to produce solation in spite of the rheopexy. Whatever the absolute quantitative meaning, however, these properties tend to confer both sensitivity and a certain kind of stabilization, and nerve cells imbedded in cardiac muscle or smooth muscle or exposed to a vascular pulse or to respiratory compression and stretch may resist solation even though thixotropic.

One further point should be noted. It is tempting to imagine that the periodic bands brought out by linear compression eflect preexisting transverse discontinu- ies of some sort in the axoplasm. This possible, but it is likely that such band- g is due to the anisotropic particles in e axoplasm being aligned by compres- on. It is well known, of course, that earing forces tend to align such parti- les, as in the case of birefringence pro- uced by flow or stretch.

The measurements of changes in light merging from the internally lighted axon uring activity are difficult to interpret. is not clear, for instance, why the hange has so far been seen in 72% ather than in 100% of the cases. In the emaining 28% it may have been too mall to detect or it may have been absent. peculation on this point is perhaps better eferred to some future time. It does eem however that, unless the whole thing an artifact, the characteristic response for less light to emerge during activity. uch a decrease might be due to a num- er of events; (a) photochemical absorp- ion in the axon, (b) increased transpar- ncy of the axoplasm allowing a dispro- ortionate amount of the light to travel long the axonal long axis thus diverting from a sideways path to the detector hotocell, (c) an increase in scattering y particulate formation in the axoplasm esulting in light undergoing more reflec- ions and so traversing a longer absorbing ath before emergence, (d) a change in efractive index of the quartz-axoplasm nterface resulting in a similar change as (b), and (e) sphericalization of a pre- iously ellipsoidal axon (Hill, '50) thus efracting the emerging light at a sharper ngle and diverting it from the solid angle ubtended by the photocell. There are not dequate experimental data to allow a de- nitive choice among the above possibili- ies at this time.

SUMMARY

1. Squid axoplasm is thixotropic, de- ormable without breaking and rheopectic. he evidence also strongly suggests that s framework elements are cross bonded nd in some way attached to the confining urface. The roles of the thixotropy and heopexy in normal physiology are dis- ussed.

2. White light emergent from the inter- ally illuminated, squid giant axon seems characteristically to decrease during activity, though this is not invariably demonstrable.

ACKNOWLEDGMENTS

The authors are deeply grateful to Dr. Robert Moon of the Department of Biophysics and to Mr. Getzold of the Central Shop for designing, fabricating and contributing an aluminum housing to allow convenient adaptation of the photocell to the microscope. Dr. Shirley Bryant kindly constructed a power supply for the photocell. Mr. Samuel Shaw helped in the execution of the experiments. The Newark Electric Co. of Chicago generously gave the laboratory the sensitive microammeter used in making the measurements.

LITERATURE CITED

Bear, R. S., F. O. Schmitt and J. Z. Young 1937 Ultrastructure of nerve axoplasm. Proc. Roy. Soc. London, B, *123:* 505–519.

Bryant, S. H., and J. M. Tobias 1955 Optical and mechanical concomitants of activity in Carcinus nerve. J. Cell. and Comp. Physiol., *46:* 71–96.

Flaig, J. 1947 Viscosity changes in axoplasm under stimulation. J. Neurophysiol., *10:* 211–221.

Frank, G. M. 1958 Physical, chemical and structural processes during initiation and propagation of impulse through the nerve fiber. Izvestia Akademii Nauk SSSR, Biol. Ser. No. 1, pp. 26–38. (In English in: The Central Nervous System and Human Behavior. Translations from the Russian Medical Literature, U. S. Dep't. of Health, Education and Welfare.)

Freundlich, H. 1935 Thixotropy. Actual. Sci. et Indust., *267:* 1.

Freundlich, H., and F. Juliusburger 1935 Thixotropy, influenced by the orientation of anisometric particles in sols and suspensions. Trans. Farad. Soc., *31:* 920–921.

Fry, W. J., and R. B. Fry 1950 A possible mechanism involved in the conduction process of thin sheathed nerves. J. Cell. and Comp. Physiol., *36:* 229–239.

Glasstone, S. 1946 Textbook of Physical Chemistry. Van Nostrand, N. Y.

Green, H., and R. N. Weltmann 1946 Thixotropy. In: Colloid Chemistry, ed. J. Alexander. Reinhold, N. Y., vol. 6, pp. 328–347.

Grundfest, H. 1936 Effects of hydrostatic pressures upon the excitability, the recovery, and the potential sequence of frog nerve. Cold. Spring Harb. Symp. Quant. Biol., *4:* 179–187.

Heilbrunn, L. V. 1943 An Outline of General Physiology. Saunders, Philadelphia.

Hill, D. K. 1950 The volume change resulting from stimulation of a giant nerve fiber. J. Physiol., *111:* 304–327.

Juliusburger, F., and A. Pirquet 1936 Thixotropy and rheopexy of V_2O_5 sols. Trans. Farad. Soc., *32:* 445–452.

Liudkovskaia, R. G. 1957 Some structural and chemical phenomena in the stimulated neurone. Biophysics, *2:* 577–589.

Philippoff, W. 1938 Über die Stromung von Stoffen mit Fliessfestigkeit. Koll. Zeit., *83:* 163–172.

Reynolds, O. 1885 On the dilatancy of media composed of rigid particles in contact. Phil. Mag., *20:* 469–481.

Shaw, S., and J. M. Tobias 1951 Preliminary measurements of birefringence changes due to electrical polarization of isolated, squid, giant axons. Biol. Bull., *101:* 229.

Spyropoulos, C. S. 1957 Response of single nerve fibers at different hydrostatic pressures. Am. J. Physiol., *189:* 214–218.

——— 1957a The effects of hydrostatic pressure upon the normal and narcotized nerve fiber. J. Gen. Physiol., *40:* 849–857.

Sutherland, W. 1905 The nature of the propagation of nerve impulse. Am. J. Physiol., *14:* 115–130.

——— 1908 The nature of the conduction of nerve impulse. Ibid., *23:* 115–130.

Tobias, J. M. 1951 Ultrastructure and function in nerve. Nonelectrical physical aspects of excitation and conduction. In: Modern Trends in Physiology and Biochemistry, ed. E. S. G. Barron. Academic Press, N. Y.

——— 1952 Some optically detectable consequences of activity in nerve. Cold Spring Harb. Symp. Quant. Biol., *17:* 15–25.

——— 1959 Biophysical aspects of conduction and transmission in the nervous system. Ann. Rev. Physiol., *21:* 299–324.

——— 1960 Further studies on the nature of the excitable system in nerve. J. Gen. Physiol., *43:* May suppl. 57–71.

Tobias, J. M., and S. H. Bryant 1955 An isolated giant axon preparation from the lobster nerve cord. J. Cell. and Comp. Physiol., *46:* 163–182.

Tobias, J. M., and P. G. Nelson 1959 Structure and function in nerve. In: A Symposium on Molecular Biology, ed. R. E. Zirkle. Univ. of Chicago Press, Chicago, pp. 248–265.

Changes in the Lipid Content During Growth and Metamorphosis of the House Fly, Musca domestica Linnaeus[1]

JOSEPH V. PEARINCOTT[2]
Department of Biology, Fordham University, New York City

Although considerable work has been done on lipid metabolism in vertebrates, not much is known of the process in invertebrates. Tichomiroff (1885) reported that during the development of the post-diapause egg of the silkworm, *Bombyx mori,* total fat content decreases with protein and glycogen, but there is an increase in the amount of lecithin. Weinland ('07) observed that during the embryonic development of the blow fly, *Calliphora vomitaria,* there is utilization of a large amount of total fat. Hill ('43) noticed that during embryogenesis of the grasshopper, *Melanoplus differentialis,* fat is the predominant energy source in late development. Rothstein ('52) showed that in the Japanese beetle, *Popillia japonica,* the total lipid remains constant during the early part of the embryonic period, but beginning with the fourth day at 30°C, when blastokinesis occurs (Gese, '53), there is a marked decrease in free lipid, continuing until the time of hatching; bound lipid on the other hand, remains constant throughout the embryonic period.

In the mature larva of the blow fly, *C. vomitaria,* Weinland ('09) noticed the fat amounts to 22% of the dry weight; in the worker of the honey bee, *Apis mellifica,* according to Strauss ('11) the corresponding value is 18%. Timon-David ('30) observed that the fat content varies widely among species, the total lipid in the larvae being 28% wet weight in the weevil, *Beloninus elephos,* and 0.94% in the cut worm, *Lycophatia margaritosa.* Finkel ('48) working on the larvae of the meal worm, *Tenebrio molitor,* found that total lipid, neutral fat, and fatty acids increase in proportion to wet weight as larval growth progresses; phospholipids decline very slowly, while total cholesterol remains practically constant. He concluded that lipid composition during larval growth of the meal worm is consistent with that known to exist in vertebrate embryos.

Changes in the lipid content during the metamorphosis of insects have been studied by several investigators. Frew ('29) observed in the blow fly, (species not given) that during the latter half of the pupal period, the fat content continually diminishes. Evans ('32) reported a rapid decrease in the fatty acids of the blow fly, *Lucillia sericata,* up to the eighth day after the larva has stopped feeding at 17°C. Following puparium formation which occurs at this time, the decrease is slower until the ninth day when synthesis of fatty acid begins. Fatty acid content reaches a maximum on the fourteenth day when histogenesis starts, after which it diminishes until the emergence of the adult on the twentieth day. Becker ('34) showed a gradual decrease in fat content during the metamorphosis of the meal worm, *T. molitor.* In this species, Evans ('34) found that fat is consumed mainly at the beginning and at the end of metamorphosis. Ludwig and Rothstein ('49) observed that in the Japanese beetle, *P. japonica,* there is a sharp drop in the ether-soluble neutral fat on the fifth and sixth days of pupal life at 25°C. From this point to the emergence of the adult (10 days after pupation), the amount of neutral fat remains relatively constant. Levenbook ('53) reported that during the metamorphosis of the blow fly, *Calliphora erythrocephala,* there is no major change in the lipid phosphorus content. Battista ('54) found that in the Japanese beetle, the total cholesterol and phospholipids increase during metamorphosis, and the fatty acid increases in the early part and decreases during the latter part of the pupal stage.

---

[1] From a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Fordham University. The author wishes to express his sincere appreciation to Dr. Daniel Ludwig under whose direction and guidance this investigation was conducted.

[2] Present address: Department of Cell Physiology, College of Physicians and Surgeons, Columbia University, New York 32, N. Y.

The above review shows that information concerning the daily changes in the lipid content of insects during development and metamorphosis is very meager, that very little work has been done on fractionation of the lipids, and that more detailed study is needed to better understand the intermediary metabolism in insects. Hence the present investigation was undertaken to demonstrate the daily changes in concentration of cholesterol, fatty acid, and lipid phosphorus during the life cycle of the house fly, *Musca domestica.*

MATERIAL AND METHODS

The house flies used in the present study were from the strain obtained from Dr. Bertram Sackter, Army Chemical Center, Maryland. The adults and larvae were fed on dog-pellets soaked in tap water, and the temperature was maintained at approximately 25°C. When the desired stage of development had been reached, the insects were weighed, and killed by placing them in small vials containing a mixture of three parts of absolute ethyl alcohol and 1 part of absolute ethyl ether (Bloor, '43). The vials with the insects were then carefully capped, labeled, and stored under refrigeration until the time of analysis. The cholesterol determinations were made using the method of Bloor ('16) which consisted of measuring the density of the green color produced by cholesterol when treated with acetic anhydride and H_2SO_4. Fatty acid analyses were based on the method of Bloor ('14) as modified by Snell and Snell ('37). This method consisted of freeing the fatty acids from tissue extract, and forming a finely dispersed precipitate in water on the addition of HCl. Turbidity of this mixture was then determined. The lipid phosphorus content was determined by the Youngburg and Youngburg ('30) technique. This method consisted of measuring the intensity of the blue color developed by the phosphorus contained in the extract when ammonium molybdate solution and aminonaphtholsulfonic acid were added. All the analyses were done using the Hellige-Diller photo-colorimeter. Lecithin values were obtained by multiplying those of lipid phosphorus by the conversion factor 25. The lipid contents were calculated as per cent wet weight, as well as milligrams per 100 individuals. Also, with the view that it will help to understand the changes in the lipids, daily determinations of the water content during the latter part of the larval and the entire pupal stage were made by weighing the insect before and after complete desiccation at 100°C. Each chemical analysis involved a mini-

TABLE 1

Changes in wet weight and water content of the house fly during growth and metamorphosis (The age of pupa is given as days after puparium formation at 25°C)

Stage	Average weight	Average water content
	mg	*% wet wt.*
Egg	0.05	—
1-day larva	1.13	—
2-day larva	1.90	—
3-day larva	14.8	82.2
4-day larva	14.3	78.8
5-day larva	19.6	77.5
6-day larva	13.4	74.5
Puparium just formed	12.3	71.6
1-day pupa	11.2	69.7
2-day pupa	10.8	69.6
3-day pupa	10.8	69.6
4-day pupa	10.2	69.5
Adult, just emerged	9.3	74.6
7 to 10-day adult	16.4	75.0

um of 10, and the analysis of the water ontent a minimum of 5 determinations.

OBSERVATIONS

The changes in wet weight and wa-er content are shown in table 1. The ge of the pupae is given as days fol-owing puparium formation. The egg has n average weight of 0.05 mg. During the arval growth the weight increases to an verage of 19.6 mg in the 5-day larva. It hen decreases to 13.4 in the 6-day larva and 12.3 mg immediately following puparium formation. During the pupal period there is a gradual decrease in the weight. The newly emerged adult has an average weight of 9.3 and the late adult (7–10 days old) of 16.4 mg. The water content of the three-day larva averages of 82.2%. It decreases steadily to 71.6% just after puparium formation, remains at approximately 69.5 during the pupal stage and increases to 75% in the adult.

TABLE 2

Changes in cholesterol content of the house fly during life cycle

Stage	Average weight of cholesterol per 100 insects	Cholesterol as per cent wet weight with standard errors
	mg	
Egg	0.010	0.201 ± 0.004
1-day larva	0.129	0.119 ± 0.002
2-day larva	0.337	0.189 ± 0.010
3-day larva	2.670	0.186 ± 0.003
4-day larva	2.470	0.177 ± 0.003
5-day larva	2.530	0.135 ± 0.002
6-day larva	2.180	0.157 ± 0.002
Puparium just formed	1.680	0.137 ± 0.003
1-day pupa	1.473	0.137 ± 0.001
2-day pupa	2.120	0.196 ± 0.001
3-day pupa	1.630	0.150 ± 0.001
4-day pupa	1.300	0.128 ± 0.001
Adult, just emerged	1.950	0.204 ± 0.002
7 to 10-day adult	2.430	0.158 ± 0.004

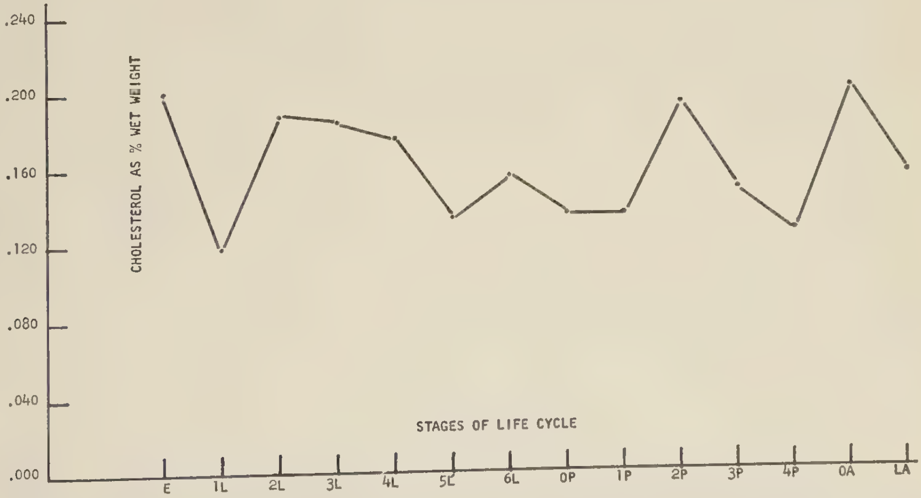


Fig. 1 Changes in the cholesterol content of the house fly during life cycle. The stages are designated as follows: E, egg; 1L through 6L, one-day-old through 6-day-old larvae; 0P through 4P, newly formed puparium through 4-day-old pupae; 0A, newly emerged adult; and LA, late adult (7–10 days old).

Figure 1 and table 2 show the changes in the cholesterol content. The values, expressed as per cent wet weight, decrease from a high of 0.201 in the egg to 0.119 in the one-day larva, then rise to 0.189 in the two-day larva, and decrease very gradually to 0.137 one day after puparium formation. During the pupal stage they fluctuate between 0.196 and 0.128, followed by an increase to 0.204 in the newly emerged, and a decrease to 0.158 in the older adults. However, in spite of these phasic decreases and increases, the overall picture shows that the percentage of cholesterol remains

TABLE 3

Changes in fatty acid content of the house fly during life cycle

Stage	Average weight of fatty acid per 100 insects	Fatty acid as per cent wet weight with standard errors
	mg	
Egg	0.13	2.67±0.054
1-day larva	2.72	2.40±0.069
2-day larva	9.70	5.39±0.150
3-day larva	97.20	6.87±0.207
4-day larva	83.20	6.04±0.147
5-day larva	101.50	5.37±0.098
6-day larva	98.00	7.11±0.111
Puparium just formed	62.40	5.22±0.083
1-day pupa	68.40	6.31±0.122
2-day pupa	55.40	5.11±0.092
3-day pupa	58.20	5.34±0.067
4-day pupa	30.00	2.96±0.061
Adult, just emerged	39.60	4.19±0.174
7 to 10-day adult	39.20	2.61±0.074

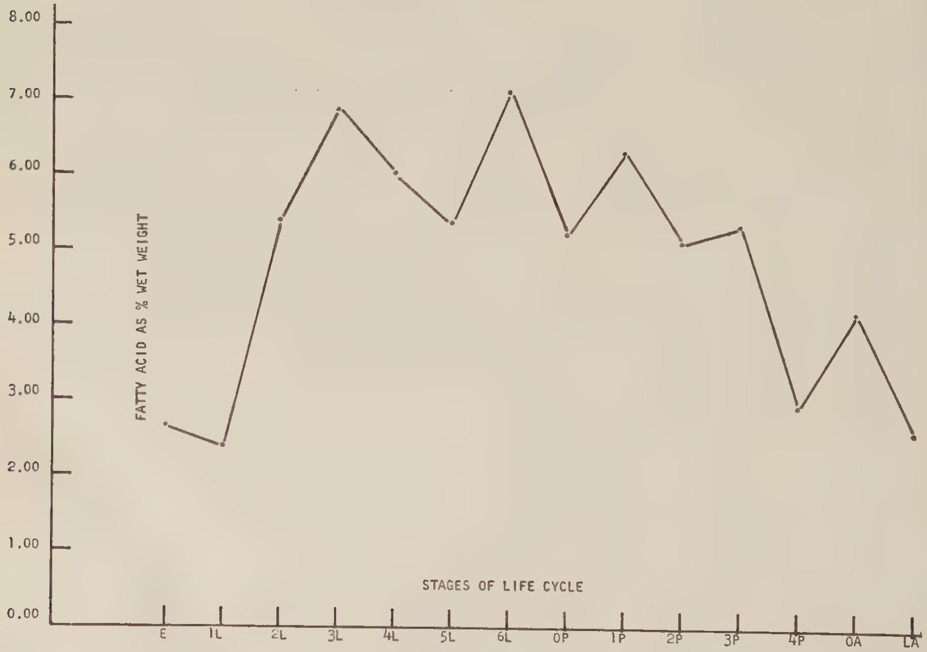


Fig. 2 Changes in the fatty acid content of the insect during life cycle. The stages are designated as follows: E, egg; 1L through 6L, one-day-old through 6-day-old larvae; 0P through 4P, newly formed puparium through 4-day-old pupae; 0A, newly emerged adult; and LA, late adult (7–10 days old).

latively constant during growth and met- norphosis.

Figure 2 and table 3 contain the results the fatty acid study. The percentage in- eases irregularly from a value of 2.67 in the egg to 7.11 in the 6-day larva. The percentage then drops progressively until the emergence of the adult although there are phasic decreases and increases. The general trend is a synthesis of fatty acids dur-

TABLE 4

Changes in lipid phosphorus content of the house fly during life cycle
(Values for lecithin were obtained by multiplying the lipid phosphorus values by 25)

Stage	Average weight of lipid phosphorous per 100 insects	Lipid phosphorus	Lecithin as per cent wet weight with standard errors
	mg	*% wet wt.*	
Egg	0.0005	0.0104	0.261 ± 0.005
1-day larva	0.0161	0.0138	0.346 ± 0.020
2-day larva	0.1496	0.0861	2.152 ± 0.054
3-day larva	0.7850	0.0535	1.328 ± 0.052
4-day larva	0.6815	0.0485	1.211 ± 0.061
5-day larva	0.6045	0.0317	0.792 ± 0.045
6-day larva	0.7530	0.0534	1.334 ± 0.053
Puparium just formed	0.2220	0.0180	0.451 ± 0.011
1-day pupa	0.1637	0.0152	0.382 ± 0.025
2-day pupa	0.1640	0.0151	0.379 ± 0.014
3-day pupa	0.1600	0.0146	0.366 ± 0.010
4-day pupa	0.0880	0.0085	0.212 ± 0.003
Adult, just emerged	0.0887	0.0092	0.229 ± 0.008
7 to 10-day adult	1.0475	0.0688	1.717 ± 0.045

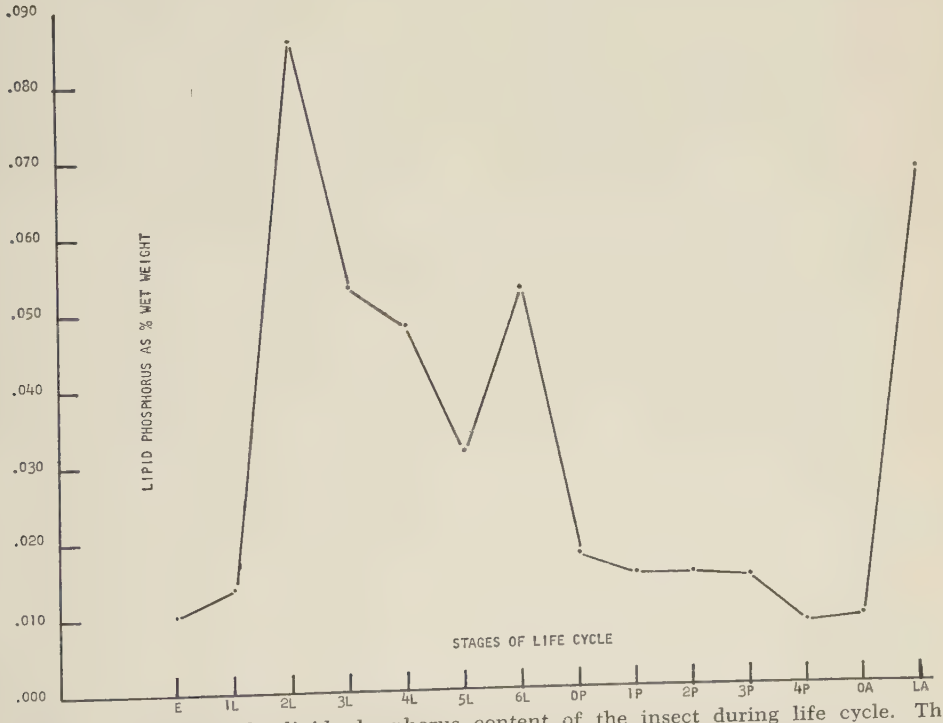


Fig. 3 Changes in the lipid phosphorus content of the insect during life cycle. The stages are designated as follows: E, egg; 1L through 6L, one-day-old through 6-day-old larvae; 0P through 4P, newly formed puparium through 4-day-old pupae; 0A, newly emerged adult; and LA, late adult (7–10 days old).

ing early larval growth and their utilization during metamorphosis.

The changes in the lipid phosphorus are shown in figure 3. Lipid phosphorus and lecithin values are given in table 4. The percentage of lipid phosphorus increases rapidly from 0.0104 to 0.0861 during the first two days of the larval period, decreases to 0.0535 during the next day, and then remains relatively constant until the end of the larval period. It decreases sharply at puparium formation, shows a very gradual decrease through the pupal period and newly emerged adult, but increases to 0.0688 in the old adult.

DISCUSSION

The constancy in the percentage of water from the first through the last day of pupal period is in accord with the findings of Ludwig and Rothstein ('49) for the Japanese beetle, *P. japonica,* in which the water content remains at approximately 78% throughout the pupal stage. However, in the newly emerged beetle the percentage decreases in contrast to the increases noted in the house fly. Hitchcock and Haub ('41) reported that in the blow fly, *Phormia regina,* the larva (within a few hours after leaving the food) and the newly emerged adult have the same percentage of water. Their results agree with the observations in the present study.

The relative constancy of the percentage of cholesterol during growth and metamorphosis of the house fly is in general agreement with the findings of Evans ('34) and Finkel ('48) for the meal worm, *T. molitor.* However, Battista ('54) observed that in the Japanese beetle there is a progressive, but not very pronounced, synthesis of cholesterol during metamorphosis. The periodic increases and decreases in the percentage values reported in the present study are similar to those shown by Evans ('34) and Finkel ('48) for the meal worm, and by Battista ('54) for the Japanese beetle. The universal presence of cholesterol in animal tissues in relatively constant amount, which is characteristic of the tissue (Bloor, Okey and Corner, '30), and the fact that it persists in fairly constant percentage during starvation (Terroine, '19) all suggest that it may be an important structural constituent of animal cells.

The building up of fatty acid during larval growth is in agreement with the findings of Finkel ('48) on *T. molitor.* The present investigation failed to show a pronounced synthesis of fatty acid during the middle of the pupal stage as reported by Evans ('32) for the blow fly, *L. sericata.* In 1934, he found that during metamorphosis of *T. molitor,* fatty acid is consumed mainly at the beginning and end, with little consumption in the middle, of the pupal period. Battista ('54) observed that during the metamorphosis of the Japanese beetle, the fatty acid content increases through the early stages, and then decreases sharply on the fifth and sixth days of pupal life at 25°C, followed by a continuous decrease until the emergence of the adult. There is an increase in glycogen during the time fatty acid decreases in the Japanese beetle (Ludwig and Rothstein, '49). In *M. domestica* no increase of fatty acid comparable with that reported by Battista during the early stages of metamorphosis has been observed. However, there is a synthesis of fatty acid during the first day after puparium formation in the house fly. It is known that fatty acids are important sources of energy during metabolic activities. Hence it is not inconceivable that a portion of the fat built up during larval growth could be used for energy during metamorphosis of insects. A few investigators (Frew, '29; Haub and Hitchcock, '41; Ludwig and Rothstein, '49) have suggested that part of the fat utilized may be converted into carbohydrate. DelVecchio ('55a) reported a synthesis of reducing compounds, expressed as glucose, during the pupal stage of the house fly, *M. domestica.* In this species, however, not enough is known about the changes in other carbohydrates during the life cycle or during metamorphosis to justify any conclusion regarding their source from fat or fatty acids.

The increase in lipid phosphorus during the first part of the larval period, followed by its relative constancy at slightly lower level during the remaining part, is in agreement with the observations of Finkel ('48) on the larval growth of the meal worm, *T. molitor.* Levenbook ('53) found that in the blow fly, *C. erythrocephala,* there is no major change in the lipid phosphorus durng metamorphosis. Battista ('54) reported hat in the Japanese beetle, the lipid phosphorus increases progressively during pupal stage. However, the rate of this rise s rather slow. DelVecchio ('55b) working on the changes in nitrogen in the house fly, *M. domestica*, showed that the lipid nitrogen decreases gradually during metamorphosis. As most of the nitrogen-containing ipids have also proportionate amount of phosphorus, the findings of the present investigation are comparable with those of DelVecchio.

SUMMARY

1. Determinations were made on the cholesterol, fatty acid, and lipid phosphorus of the house fly during the following stages: egg, larvae on each day of larval period, pupae on each day of pupal life, and newly emerged and 7–10 days old adults. Water content was determined during all the above stages except the egg and the early part of the larval stage.

2. The water content decreases steadily from 82.2 in the three-day larva to 69.7% wet weight in the one-day pupa. It remains approximately constant throughout the rest of the pupal period, and then increases to approximately 75% in the adult.

3. The percentage of cholesterol, although it showed phasic increases and decreases, remains relatively constant throughout the life cycle at an average value of 0.160.

4. The percentage of fatty acid increases from 2.40 in the one-day to 6.87 in the three-day larva, remains approximately steady during the rest of the larval period, and then decreases progressively to 2.96 in the 4-day pupa. The values are 4.19 in the newly emerged, and 2.61 in the old adults.

5. The percentage of lipid phosphorus increases rapidly from 0.0138 in the one-day to 0.0535 in the three-day larva. It remains relatively constant during the remainder of the larval stage, and then decreases sharply to 0.018 during puparium formation, followed by a very gradual decrease through the pupal period and through the newly emerged adult. The value increases sharply in the late adult to 0.0688.

6. The results of the present investigation indicate that cholesterol is probably a tissue constituent rather than an energy source; that fatty acid is an important source of energy during metamorphosis; that the phospholipids increase along with the increase of fatty acid during larval growth, and that they show a very gradual decrease during the pupal period as compared with a rapid decrease in fatty acid.

LITERATURE CITED

Battista, G. W. 1954 Changes in the fat content of the Japanese beetle, *Popillia japonica* Newman, during metamorphosis. J. N. Y. Ent. Soc., *62:* 27–37.

Becker, M. 1934 Wandlungen des Fettes während der Metamorphose. Biochem. Ztschr., *272:* 227–234.

Bloor, W. R. 1914 A method for the determination of fat in small amounts of blood. J. Biol. Chem., *17:* 377–384.

——— 1916 The determination of cholesterol in blood. Ibid., *24:* 227–231.

——— 1943 Biochemistry of the Fatty Acids and their Compounds, the Lipids. New York.

Bloor, W. R., R. Okey and G. W. Corner 1930 The changes in the lipid content of the corpus luteum of the sow. J. Biol. Chem., *86:* 291–306.

DelVecchio, R. J. 1955a Changes in the concentration of reducing substances during the metamorphosis of the housefly, *Musca domestica* L. J. N. Y. Ent. Soc., *63:* 9–15.

——— 1955b Changes in the distribution of nitrogen during growth and metamorphosis of the housefly, *Musca domestica* L. Ibid., *63:* 141–152.

Evans, A. C. 1932 Some aspects of chemical changes during insect metamorphosis. J. Exp. Biol., *9:* 314–322.

——— 1934 On the chemical changes associated with metamorphosis in the beetle, *Tenebrio molitor* L. Ibid., *11:* 397–401.

Finkel, A. J. 1948 The lipid composition of *Tenebrio molitor* larvae. Physiol. Zool., *21:* 111–133.

Frew, J. G. H. 1929 Studies in the metabolism of insect metamorphosis. Brit. J. Exp. Biol., *6:* 205–218.

Gese, E. C. 1953 Embryogeny of the Japanese beetle, *Popillia japonica* Newman, and a correlation of morphological events with known physiological changes. Ph.D. thesis, New York University.

Haub, J. G., and F. A. Hitchcock 1941 The interconversion of foodstuffs in the blowfly, *Phormia regina*, during metamorphosis. III. Chemical composition of larvae, pupae, and adults. Ann. Ent. Soc. America, *34:* 32–37.

Hill, D. L. 1943 Certain relationships between carbohydrate and respiratory metabolism during embryonic development (Orthoptera). Anat. Rec., *87:* 21–22.

Hitchcock, F. A., and J. G. Haub 1941 The interconversion of foodstuffs in the blowfly, *Phormia regina*, during metamorphosis. I. Respiratory metabolism and nitrogen excretion. Ann. Ent. Soc. America, *34:* 17–25.

Levenbook, L. 1953 The variation in phosphorus compounds during metamorphosis of the blowfly, *Calliphora erythrocephala* Meig. J. Cell. and Comp. Physiol., *41:* 313–334.

Ludwig, D., and F. Rothstein 1949 Changes in the carbohydrate and fat content of the Japanese beetle, *Popillia japonica* Newman, during metamorphosis. Physiol. Zool., *22:* 308–317.

Rothstein, F. 1952 Biochemical changes during the embryonic development of the Japanese beetle, *Popillia japonica* Newman. Ibid., *25:* 171–178.

Snell, F. D., and C. T. Snell 1937 Colorimetric Methods of Analysis. New York.

Strauss, J. 1911 Die chemische Zusammensetzung der Arbeitsbienen und Drohnen während ihrer verschiedenen Entwicklungstudien. Ztschr. f. Biol., *56:* 347–397.

Terroine, E. F. 1919 Contribution à la connaissance de la physiologie des substances grasses et lipoidiques. Paris.

Tichomiroff, A. 1885 Chemische Studien über die Entwicklung der Insecteneier. Ztschr. f. Physical. Chem., *9:* 518–532.

Timon-David, J. 1930 Contribution à l'étude de la biochimie entomologique. Huilles et graisses d'insectes. Bull. Soc. Chim. Biol., *12:* 395–403.

Weinland, E. 1907 Weitere Beobachtungen an *Calliphora.* IV. Über chemische Momente bei der Metamorphose und Entwicklung. Ztschr. f. Biol., *49:* 486–493.

——— 1909 Chemische Beobachtungen an der Fliege *Calliphora.* Biol. Zentralbl., *29:* 564–577.

Youngburg, G. E., and M. V. Youngburg 1930 A system of blood phosphorus analysis. J. Lab. Clin. Med., *16:* 159–166.

The Effect of Tannic Acid on the Permeability of Erythrocytes to Non-electrolytes

F. R. HUNTER[1]
Department of Biology, University of the Andes, Bogotá, Colombia

Since tanning agents have been most widely used in the preparation of hides, it is not surprising to find that a large number of studies have been made concerning the effect not only of the vegetable tannins but also of substances such as chromium and aldehydes on the protein, collagen. The chemical aspects of these reactions are discussed by Gustavson in a review article ('49) and in a book ('56) and also by several authors in Discussion no. 17 of the Faraday Society ('54) (Schulman and Dogan; Ellis and Pankurst; (Gustavson). The reactions between a vegetable tannin, such as tannic acid, and collagen generally seem to involve both electrovalent and coordinate forces. Since the formation of additional cross linkages can so markedly change the properties of collagen, it is suggested that by treating cell membranes with a tanning agent, the proteins of the membrane might be similarly altered. If additional cross linkages could be formed in the cell membrane, it might be possible to demonstrate a decrease in the size of the "pores."

That tannic acid does indeed change certain properties of the erythrocyte membrane is indicated by immunological experiments in which tanned erythrocytes react differently in certain antigen-antibody reactions than they did prior to this treatment (e.g., Boyden, '51; Feinberg and Flick, '54). There are also experiments to show that the rate of movement of ions across the eyrthrocyte membrane is greatly decreased in the presence of tannic acid (Jacobs et al., '43; Edelberg, '52). Jacobs, however, did not observe much effect of this treatment on the rate of movement of non-electrolytes. Edelberg ('53) studied model systems to identify the reaction responsible for the decrease in ion permeability and concluded that his data were consistent with the view that ions entered through a loci predominantly protein in nature.

The present experiments were undertaken in an attempt to extend and clarify the observations concerning non-electrolyte permeability of tanned erythrocytes. It is hoped that such a study may add to our understanding of the relationship between the proteins in the cell membrane and the movement of non-electrolytes across it.

GENERAL PROCEDURES

Two series of experiments were performed. In one series erythrocytes were suspended in a solution containing tannic acid and an aliquot of these cells was removed at some subsequent time for testing their permeability. In the other series, erythrocytes were added to a solution containing the molecule whose permeability was to be tested plus tannic acid. The first series, then, may be considered a "chronic" type of experiment while the second series was an "acute" experiment.

Studies included blood from chickens, rabbits (drawn by cadiac puncture) and humans (cubital vein). In the majority of experiments heparin was used as an anticoagulant although in some instances oxalate was used with human blood with similar results. The cells were washed twice with Ringer Locke solution (R.L.) and the supernatant fluid and buffy layer were removed by aspiration.

Hematocrit measurements were made using an air turbine (Parpart and Ballentine, '43). A photocell, D.C. amplifier and pen recorder apparatus (Mawe, '56) was used to measure permeability. Both the rate of penetration and the rate of exit of molecules were measured (cf. Hunter, '53). A glass electrode was used to measure pH and a Beckman spectrophotometer (545 mμ) to measure the per cent of hemoglobin in supernatant solutions. All centrifugations were made at a speed of 1500 x g or less. The permeability measurements were made at room temperature (22–25°C).

---

[1] Acknowledgment is made to the Rockefeller Foundation and to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the support of this research.

Chronic experiments—chicken blood

Equal volumes of cells and R.L. were mixed for a control suspension and equal volumes of cells and tannic acid in R.L. were mixed for an experimental suspension. In the majority of the experiments, the tannic acid solution was freshly prepared and the pH was adjusted to approximately 7 by the addition of sodium bicarbonate.

The rates of penetration of water, ethylene glycol and glycerol were measured using the hemolysis technique. In the majority of experiments, 0.1 cm^3 of the cell suspension was added to 10 cm^3 of water or to 10 cm^3 of a 0.3 M solution of the penetrating molecule. Also, to study the rate of penetration, cells were added (0.05–0.2 cm^3, depending on the sensitivity setting of the amplifier) to 10 cm^3 of a 0.3 M solution of the penetrating molecule dissolved in R.L. To study the rate at which molecules diffused from the cells, equal volumes of the stock erythrocyte suspensions and a solution of 0.6 M non-electrolyte in R.L. were equilibrated. An aliquot (0.1–0.4 cm^3, depending on the sensitivity setting of the amplifier) of these new suspensions was then added to 10 cm^3 of a 1.5 × R.L. and the rate of shrinking was measured.

In different experiments, cells were mixed with equal volumes of 1:20,000–1:500 tannic acid in R.L. In subsequent descriptions of chronic experiments, the concentrations of the tannic acid solutions refer to the concentrations of the solutions that were mixed with the cells unless otherwise stated. Measurements were made at times varying from immediately after mixing the cells with the tannic acid up to 9 hours after mixing. In some experiments, measurements were made the following day after the cell suspensions had been placed in the refrigerator overnight (in one experiment, a total of 28 hours after mixing the cells with the tannic acid—9 hours in the refrigerator plus 21 hours at room temperature).

To avoid unnecessary details, table 1 presents the averages of the times for one half the total deflection of the pen recorder regardless of the concentration of tannic acid or the time of exposure of the cells to this solution. The first column of the table indicates the method and solution

TABLE 1

The effect of tannic acid on the permeability of chicken erythrocytes

Method	Concentration tannic acid	No. of experiments	Time in seconds for one half total deflection	
			Control	Experimental
Hemolysis in water	1:10,000	14	19.8 ± 0.2	
	1:500	12		19.7 ± 0.3
Hemolysis in ethylene glycol	1:20,000			
	1:10,000	19	33.5 ± 0.7	33.1 ± 0.9
	1:5,000			
	1:500			
Swelling in ethylene glycol	1:10,000	14	8.2 ± 0.5	7.6 ± 0.4
Swelling in glycerol	1:500	9	258 ± 21	
		11		253 ± 13
Shrinking in ethylene glycol	1:10,000	10	6.0 ± 0.2	
	1:5,000	12		6.2 ± 0.2
	1:500			
Shrinking in glycerol	1:500	17	203 ± 11	
		22		246 ± 14

TABLE 2

The effect of tannic acid on the relative volumes (hematocrits) of chicken erythrocytes

Concentration tannic acid	Hours of exposure	Hematocrit	
		Control	Experimental
1:20,000	0	41.3	41.1
	4	43.2	41.7
	7	42.4	41.4
1:10,000	0	40.9	40.3
	2	41.5	41.6
	7	47.0	49.4
1:5,000	0	38.7	42.5
	2	39.6	42.4
	4	39.2	42.8
	9	39.5	43.5
	22	40.5	46.5
	26	48.3	50.4
1:1,000	0	43.2	44.2
	2	44.4	43.6
1:500	0	44.2	47.6
	1½	43.0	46.9
	3	43.7	47.4
1:500	0	29.2	31.8
	11	30.2	32.3

TABLE 3

The rate of shrinking of chicken erythrocytes in 1.5 × Ringer Locke solution after previous equilibration in 0.6 M glycerol in Ringer Locke solution

Tannic acid concentration	No. of experiments	Time in seconds for one half total deflection		t-values
		Control	Experimental	
1:20,000	14	225 ± 13	231 ± 12	0.3
1:20,000	13	226 ± 8		
	12		232 ± 7	0.5
1:10,000	9	202 ± 6		
	11		188 ± 7	1.5
1:1,000	14	231 ± 6		
	15		289 ± 8	5.6
1:1,000	10	188 ± 7		
	11		243 ± 9	4.7
1:1,000	8	165 ± 2	195 ± 7	4.2
1:500	7	189 ± 11		
	12		295 ± 15	5.8
1:500	6	142 ± 4		
	3		205 ± 4	11.9
1:500	9	140 ± 3		
	8		220 ± 6	11.9

used, the second, the concentrations of tannic acid to which the cells were exposed in the various experiments, the third, the number of experiments and the 4th and 5th, the times including the standard errors of the means. In table 2 are volume measurements of these same cells after they had stood in the stock suspensions for different periods of time. The data in table 2 show that cells both in the presence of R.L. and in the presence of tannic acid in R.L. swell slightly (cf. Hunter et al., '56) but that there is no consistent difference between the volume changes of the control and experimental cells.

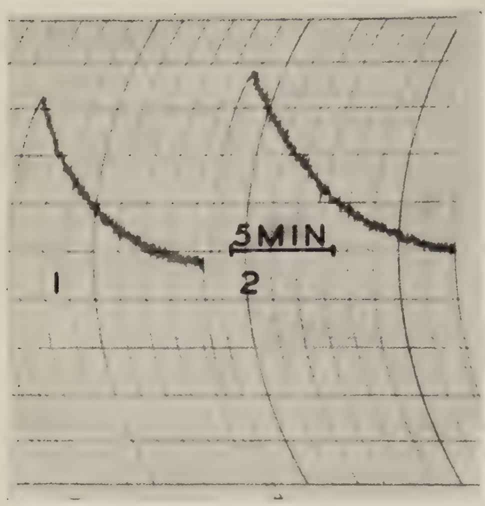


Fig. 1 The effect of 1:1000 tannic acid on the rate of shrinking of chicken erythrocytes in 1.5 × R.L. following equilibration in 0.6 M glycerol in R.L. 1, control; 2, experimental.

In view of the data in table 1, a more detailed study was made of the rate of shrinking in glycerol of these cells. These data are presented in table 3. The significance of the differences between control and experimental values was calculated using the *t* tables of Fisher. These values are included in the last column of the table. It can be seen that tannic acid in a concentration of 1:1000 or 1:500 very significantly slows the rate of exit of glycerol from these cells. A typical pair of curves is shown in figure 1.

Chronic experiments—rabbit blood

Similar experiments were performed using rabbit blood. These data, presented in table 4 show essentially no effect of tannic acid. The difference between the rate of shrinking in glycerol of the control and experimental cells is only significant at the 10% level.

Chronic experiments—human blood

Since Jacobs and Stewart (unpublished) and Edelberg ('52) had found an effect of tannic acid on the rate of penetration of glycerol into erythrocytes of humans and pigeons under appropriate conditions, this point was checked using human blood. From table 5 it can be seen that glycerol enters and leaves human erythrocytes at a rate very significantly different from control cells. To test whether or not this effect of tannic acid could be reversed, experiments were performed in which the stock suspensions were washed twice with R.L. and left in the refrigerator overnight. The rate of swelling in 0.3 M glycerol in R.L. was measured the next day. The average time in seconds for half the total deflection was 8.7 for 11 controls and 8.7 for 9 experimentals suggesting that the inhibition had been reversed.

Acute Experiments

Since the Pennsylvania group had used the hemolysis technique, it seemed of interest to repeat some of their observations using the swelling and shrinking tech-

TABLE 4

The effect of tannic acid on the permeability of rabbit erythrocytes

Method	Concentration tannic acid	No. of experiments	Time in seconds for one half total deflection	
			Control	Experimental
Hemolysis in water	1:500	2	2	3
Hemolysis in ethylene glycol	1:500	2	3	
		3		3
Hemolysis in glycerol	1:500	3	37	38
Swelling in 0.3 M glycerol	1:500	10	7.0 ± 0.9	
		9		6.3 ± 0.8
		10	11.7 ± 0.5	10.1 ± 0.4
Swelling in 0.6 M glycerol	1:500	4	17.5 ± 1.0	
		5		15.2 ± 0.9
Shrinking in glycerol	1:500	13	7.4 ± 0.6	
		14		9.0 ± 0.7

TABLE 5

The effect of tannic acid on the permeability of human erythrocytes

Method	Concentration tannic acid	No. of experiments	Time in seconds for one half total deflection		t-value
			Control	Experimental	
Swelling in 0.6 M glycerol	1:1,000	26	10.6±0.45		
		30		16.5±0.73	6.9
Shrinking in 1.5 × R.L. following equilibration in 0.6 M glycerol	1:1,000	36	6.4±0.26		
		32		7.9±0.34	3.6

niques and to extend these observations to a more detailed study of a possible effect of tannic acid on non-electrolyte permeability. Edelberg emphasized the problem of the loss of tannic acid by absorption on glassware. To avoid this loss he coated all of his glassware with silicone. His cell suspensions were placed in his densimeter only for a short time to measure his end point. In the present experiments the cell suspensions were in the densimeter for much longer periods of time in order to measure the whole course of the volume changes of the cells. Since it was impractical to coat the chamber of the densimeter with silicone, higher concentrations of tannic acid were used in the present experiments than in those of Edelberg. With this exception, Edelberg's procedures were followed. In general, the erythrocytes were washed twice with R.L. buffered with phosphate to pH 7.4 (as in Edelberg's work—2% by volume M/8 Na_2HPO_4–NaH_2PO_4 containing 6×10^{-5} M $NaHCO_3$). Erythrocytes were then added to a solution of the penetrating molecule in the densimeter (control) or to the same solution containing tannic acid (experimental). A comparison of the rates of swelling of the cells in these two solutions should indicate whether or not the tannic acid had an effect on the rate of penetration of that molecule. After each record was obtained, the suspension was removed from the densimeter, the pH was measured, and the suspension centrifuged. The supernatant fluid was saved for subsequent measurement of the amount of hemolysis. The cells were then added to an appropriate dilution of R.L. (0.7–1.75 × R.L. depending on the concentration of the penetrating molecule in the cells) in the densimeter and the curve showing the rate of shrinking was obtained. A comparison of these two curves should indicate whether or not the tannic acid had any effect on the rate of exit of the penetrated molecule. After each record was obtained, this suspension was removed, the pH measured, and the suspension centrifuged. This supernatant fluid was saved for determination of the amount of hemolysis. At this point an attempt was made to remove the tannic acid from the experimental cells. At first Edelberg's method of using gelatin in R.L. (0.4 cm^3 of 1% gelatin in R.L. added to 10 cm^3 R.L.) was tried. This treatment was quite effective in reversing the tannic acid effect (cf. fig. 5) but it also increased the fragility of both control and experimental cells (cf. Edelberg, '52, p. 536). Consequently in other experiments the cells were washed twice with R.L. which also was quite effective in reversing the action of tannic acid. In still other experiments the cells were not washed since the test solutions in the densimeter essentially served to wash the cells.

Following one of these three treatments, the cells were again added to the solution of the penetrating molecule in the densimeter and the rate of swelling was again measured. A comparison of the rate of swelling of the control and experimental cells should show whether or not the tannic acid effect was reversed. The suspension was again removed, the pH measured, and the suspension centrifuged. This supernantant fluid was also saved for hemoglobin measurements. The cells were then added to the appropriate dilution of R.L. in the densimeter and the rate of exit of the molecules was measured to see if the tannic acid effect on the rate of exit had been reversed. That is to say,

TABLE 6

Summary of the acute experiments showing the effect of tannic acid on the permeability of erythrocytes

Type of erythrocyte	Solution first used	Concentration R.L. used for shrinking	Final concentration tannic acid (most concentrated used)	Comments
Human	M/6 NH_4Cl in R.L.-PO_4	1.5 ×	3×10^{-3}	Inhibition and reversal Similar to fig 2 but much less inhibition
	0.3 M glycerol in R.L.	1.5 ×	6×10^{-3}	Inhibition and reversal Similar to fig. 3
	0.05 M erythritol in R.L.	0.7 ×	6×10^{-3}	Inhibition and reversal—fig. 5
Rabbit	M/6 NH_4Cl in R.L.-PO_4	1.5 ×	6×10^{-3}	Inhibition and reversal—fig. 2
	0.3 M glycerol in R.L.	1.5 ×	$2.5\text{–}7.5 \times 10^{-2}$	Inhibition and reversal—fig. 3
	0.6 M ethylene glycol in R.L.	1.75 ×	5×10^{-2}	Slight inhibition and reversal
			1×10^{-1}	Similar to fig. 2 but only very slight inhibition
	0.05 M erythritol in R.L.	0.7 ×	6×10^{-3}	Inhibition and reversal Similar to fig. 5 but less inhibition
Chicken	0.6 M ethylene glycol in R.L.	1.75 ×	2.4×10^{-2}	Slight inhibition and reversal Similar to fig. 2 but only very slight inhibition
	0.3 M glycerol in R.L.	1.5 ×	1.8×10^{-2}	Inhibition and reversal—fig. 4
			2.4×10^{-2}	

the same cells were used for 4 measurements in the densimeter. Tannic acid was present only in the solution used for the first of these 4 measurements. However, the tannic acid had not been removed from the cells when they were added to the second solution.

To simplify the presentation of the results, the types of experiments performed are summarized in table 6 and typical records are included in figures 2–5. In the legend of each of these figures are included the details of the procedures.

It can be seen from table 6 that in every case studied, tannic acid in sufficiently high concentrations, decreased the rate of movement of molecules across the membrane of the three different types of erythrocytes. In general, higher concentrations of tannic acid gave more inhibition and the larger the penetrating molecule, the more obvious was the inhibition.

DISCUSSION

Unfortunately, there are various factors which might lead one to question the interpretation of these results. The most disturbing of these is the tendency of tanned erythrocytes to agglutinate (see, for example, Edelberg, '52 and Feinburg and Flick, '54). With the concentrations of tannic acid used in the acute experiments the agglutination of the cells was very obvious. Hoffman ('52) has shown that changes in agglutination of a suspension of erythrocytes of various species and also the disc-sphere transformation (Hoffman et al., '55) can change the amount of light passing through a suspension of erythrocytes. Considering the chronic experiments first, the following microscopic observations were made. Stock suspensions of cells in R.L. and of cells in 1:500 tannic acid in R.L. were prepared. A few aggregates of cells were observed in the

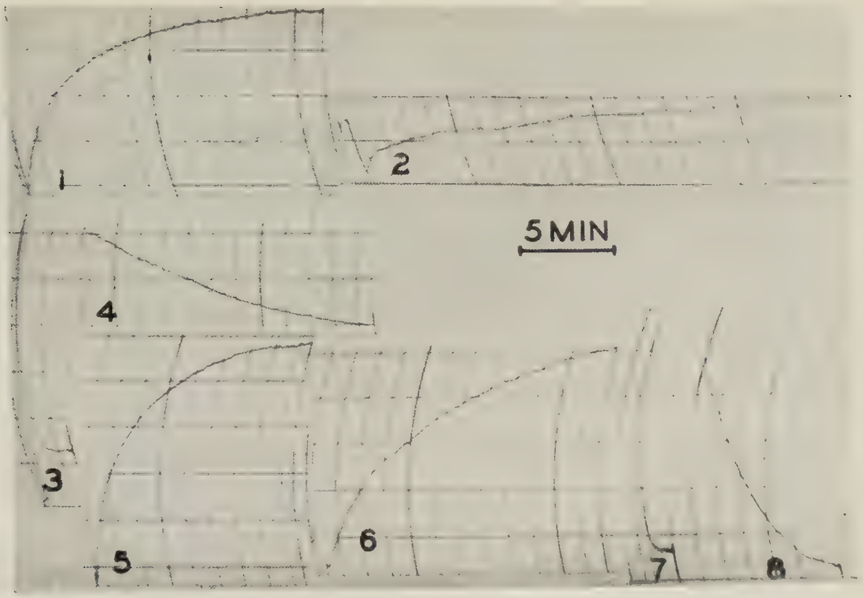


Fig. 2 The effect of tannic acid on the rate of movement of anions into and out of rabbit erythrocytes. (Final concentration of tannic acid $= 6 \times 10^{-3}\%$.) 1, Rate of swelling of control cells in NH_4Cl. 2, Rate of swelling of experimental cells showing inhibition due to tannic acid. 3, Rate of shrinking of controls. 4, Rate of shrinking of experimentals showing tannic acid inhibition. 5, 6, Swelling of control and experimental cells respectively showing partial reversal of tannic acid inhibition. 7, 8, Shrinking of control and experimental cells respectively showing partial reversal of tannic acid effect.

1. 1 cm^3 R.L.–phosphate plus 9 cm^3 M/6 NH_4Cl in R.L. plus 0.05 cm^3 erythrocytes.
2. 1 cm^3 R.L.–phosphate plus 8 cm^3 M/6 NH_4Cl in R.L. plus 1 cm^3 M/6 NH_4Cl in R.L. containing 0.6 mg tannic acid (total amount of TA.) plus 0.05 cm^3 erythrocytes.
3. 10 cm^3 1.5 × R.L. plus cells from no. 1.
4. 10 cm^3 1.5 × R.L. plus cells from no. 2. The cells from no. 3 and no. 4 were washed twice with R.L.
5. 10 cm^3 M/6 NH_4Cl in R.L. plus cells from no. 3.
6. 10 cm^3 M/6 NH_4Cl in R.L. plus cells from no. 4.
7. 10 cm^3 1.5 × R.L. plus cells from no. 5.
8. 10 cm^3 1.5 × R.L. plus cells from no. 6.

tannic acid suspension. These suspensions were then diluted 1:100 (the dilution used in the densimeter). As was true with the more concentrated suspensions, there were a few aggregates in the control and many more in the experimental suspension. The stock suspensions were then diluted 1:100 with 0.3 M glycerol in R.L. and observed immediately after mixing and after a sufficient time interval for the glycerol to have entered the cells. The control solution had no aggregates at first and only a few after the cells had changed volume. In the experimental solution there were a few aggregates at first but the number of aggregates did not change after the cells had changed volume.

Similar observations were made in all of the experiments in which 1:1000 tannic acid was used. The initial control and experimental stock suspensions were observed microscopically. Observations were also made on the stock suspensions of these cells mixed with 0.6 M glycerol in R.L. and also at the beginning and at the end of shrinking measurements in the densimeter. In one experiment, no agglutination was seen in any of the above solutions. In other experiments a few small clumps (no more than three cells per clump) were observed in the various experimental solutions. But the number of these clumps at the beginning and at the end of a swelling experiment were the same. These observations would sug-

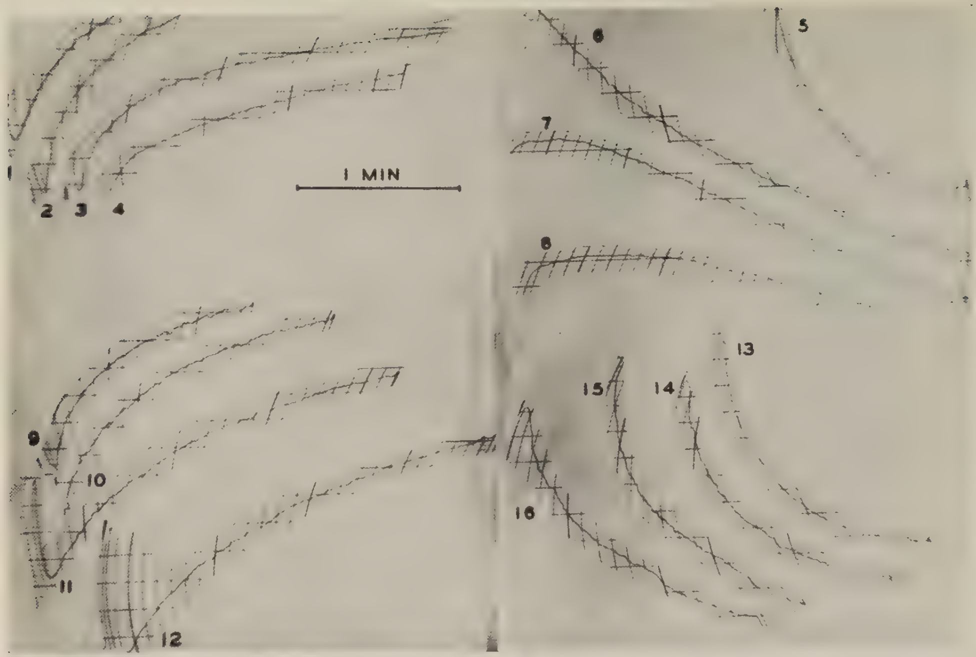


Fig. 3 The effect of increasing concentrations of tannic acid on the permeability of rabbit erythrocytes to glycerol. 1, 5, 9 and 13, control. 1, 9, rate of swelling in glycerol; 5, 13, rate of shrinking as glycerol leaves the cells. 2, 6, 10, 14, experimental; $2.5 \times 10^{-2}\%$ tannic acid final concentration. 3, 7, 11, 15, experimental; $5.0 \times 10^{-2}\%$ tannic acid final concentration. 4, 8, 12, 16, experimental; $7.5 \times 10^{-2}\%$ tannic acid final concentration.

1. 10 cm^3 0.3 M glycerol in R.L. plus 0.04 cm^3 cells.
2. 9 cm^3 0.3 M glycerol in R.L. plus 1 cm^3 0.3 M glycerol in R.L. containing 2.5 mg tannic acid plus 0.04 cm^3 cells.
3. 8 cm^3 0.3 M glycerol in R.L. plus 2 cm^3 0.3 M glycerol in R.L. containing 5.0 mg tannic acid plus 0.04 cells.
4. 7 cm^3 0.3 M glycerol in R.L. plus 3 cm^3 0.3 M glycerol in R.L. containing 7.5 mg tannic acid plus 0.04 cm^3 cells.
5. 10 cm^3 1.5 × R.L. plus cells from no. 1.
6. 10 cm^3 1.5 × R.L. plus cells from no. 2.
7. 10 cm^3 1.5 × R.L. plus cells from no. 3.
8. 10 cm^3 1.5 × R.L. plus cells from no. 4.

The following cells were not washed. The resuspension in 1.5 × R.L. and glycerol in R.L. served to partially reverse the tannic acid effect.

9. 10 cm^3 0.3 M glycerol in R.L. plus cells from no. 5.
10. 10 cm^3 0.3 M glycerol in R.L. plus cells from no. 6.
11. 10 cm^3 0.3 M glycerol in R.L. plus cells from no. 7.
12. 10 cm^3 0.3 M glycerol in R.L. plus cells from no. 8.
13. 10 cm^3 1.75 × R.L. plus cells from no. 9.
14. 10 cm^3 1.75 × R.L. plus cells from no. 10.
15. 10 cm^3 1.75 × R.L. plus cells from no. 11.
16. 10 cm^3 1.75 × R.L. plus cells from no. 12.

gest that even though the tanned cells might tend to form small clumps, a change in the degree of agglutination could not explain the light changes observed during the shrinking experiments. No change comparable to a disc-sphere transformation was observed in any of these suspensions. Another type of observation also substantiates the idea that neither changes in agglutination nor shape changes could account for the light changes in the densimeter. Figure 6 shows records obtained when cells exposed to 1:1000 tannic acid and control cells are suspended in R.L. as compared with swelling in 0.3 M glycerol in R.L. and shrinking in 1.5 × R.L. of the same suspension. It is difficult to understand

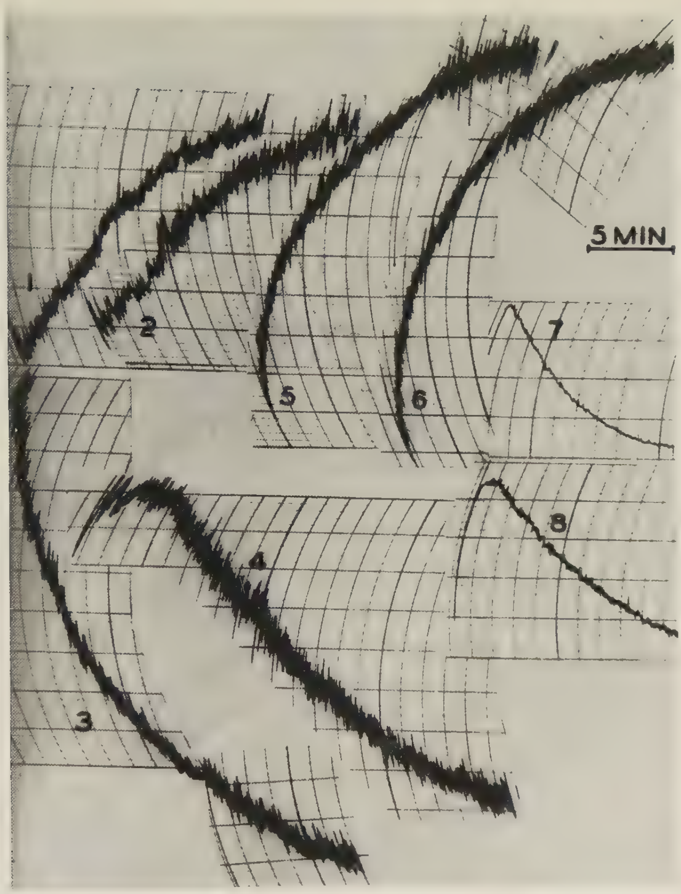


Fig. 4 The effect of tannic acid on the permeability of chicken erythrocytes to glycerol (final concentration of tannic acid = $1.8 \times 10^{-2}\%$). 1, Rate of swelling of control cells in glycerol. 2, Rate of swelling of experimental cells showing slight inhibition due to tannic acid. 3, Rate of shrinking of controls. 4, Rate of shrinking of experimentals showing inhibition. 5,6, Swelling of control and experimental cells respectively showing reversal of inhibition (larger deflection of pen recorder due to small amount of hemolysis resulting from increased fragility of cells). 7, 8, Shrinking of control and experimental cells respectively showing partial reversal of inhibition (sensitivity of amplifier was decreased).

1. 10 cm^3 0.3 M glycerol in R.L. plus 0.05 cm^3 cells.
2. 7 cm^3 0.3 M glycerol in R.L. plus 3 cm^3 0.3 M glycerol in R.L. containing 1.8 mg. T.A. plus 0.05 cm^3 cells.
3. 10 cm^3 1.5 × R.L. plus cells from no. 1.
4. 10 cm^3 1.5 × R.L. plus cells from no. 2.

The following cells were not washed:

5. 10 cm^3 0.3 M glycerol in R.L. plus cells from no. 3.
6. 10 cm^3 0.3 M glycerol in R.L. plus cells from no. 4.
7. 10 cm^3 1.5 × R.L plus cells from no. 5.
8. 10 cm^3 1.5 × R.L. plus cells from no. 6.

how the small, random fluctuations in the R.L. could systematically change the much larger light changes observed in the other two solutions.

Microscopic observations were not made of the cells in the acute experiments because in many of the experiments in which the higher concentrations of tannic acid were used it was obvious from the rate at which the cells settled out in the centrifuge tubes that agglutination had occurred. That this could not account for

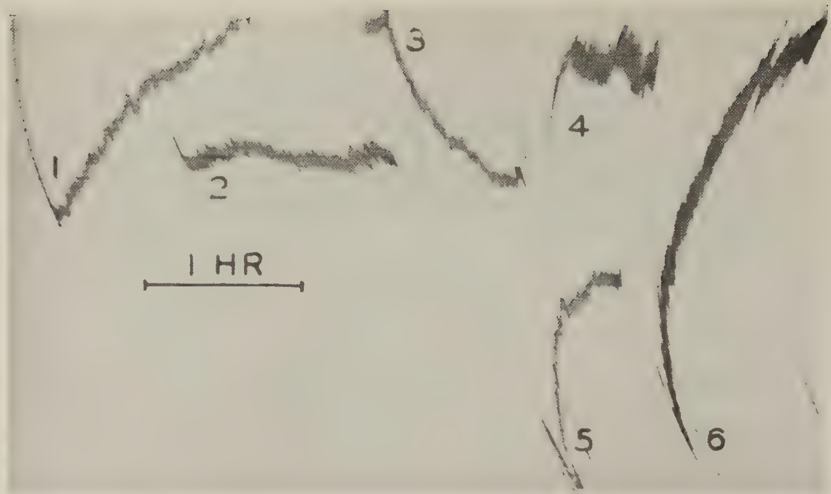


Fig. 5 The effect of tannic acid on the permeability of human erythrocytes to erythritol. (Final concentration of T.A. = $6 \times 10^{-3}\%$). 1, Rate of swelling of control cells in erythritol. 2, Rate of swelling of experimental cells showing complete inhibition. 3, Rate of shrinking of controls. 4, Lack of shrinking of experimentals. 5,6, Swelling of control and experimental cells respectively showing reversal of inhibition.

1. 10 cm^3 0.05 M erythritol in R.L.–PO_4 plus 0.04 cm^3 cells.
2. 9 cm^3 0.05 M erythritol in R.L.–PO_4 plus 1 cm^3 0.05 M erythritol in R.L.–PO_4 containing 0.6 mg T.A. plus 0.04 cm^3 cells.
3. 10 cm^3 0.7 × R.L.–PO_4 plus cells from no. 1.
4. 10 cm^3 0.7 × R.L.–PO_4 plus cells from no. 2.

Cells washed with gelatin:

5. 10 cm^3 0.05 M erythritol in R.L.–PO_4 plus cells from no. 3.
6. 10 cm^3 0.05 M erythritol in R.L.–PO_4 plus cells from no. 4.

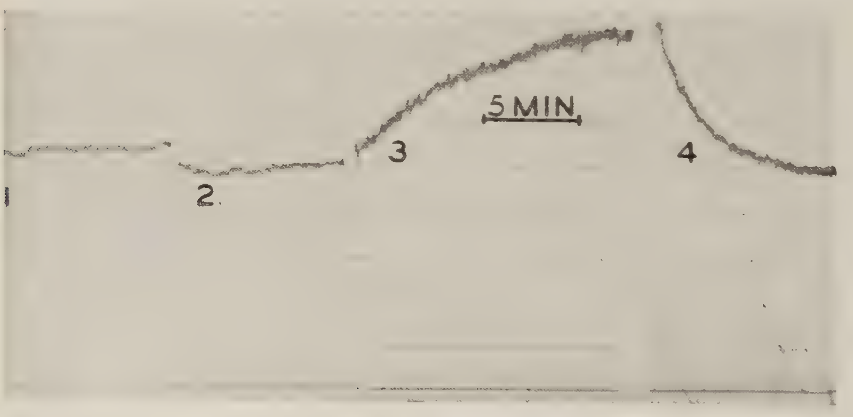


Fig. 6 A comparison of the light changes observed when chicken erythrocytes are suspended in different solutions. 1, 0.08 cm^3 experimental cells (1 : 1000 tannic acid) plus 10 cm^3 R.L. 2, Same, except control cells. 3, 0.08 cm^3 control cells plus 10 cm^3 0.3 M glycerol in R.L. 4, 0.15 cm^3 control cells in 0.6 M glycerol in R.L. plus 10 cm^3 1.5 × R.L.

the differences in rate of the apparent swelling and shrinking of the cells is suggested by figure 7. Here it can be seen that the rate of movment of ethylene glycol into and out of rabbit erythrocytes is unaffected by a concentration of tannic acid of 2.4×10^{-2}. This concentration of tannic acid agglutinates the cells sufficiently so that they settle out rapidly on standing.

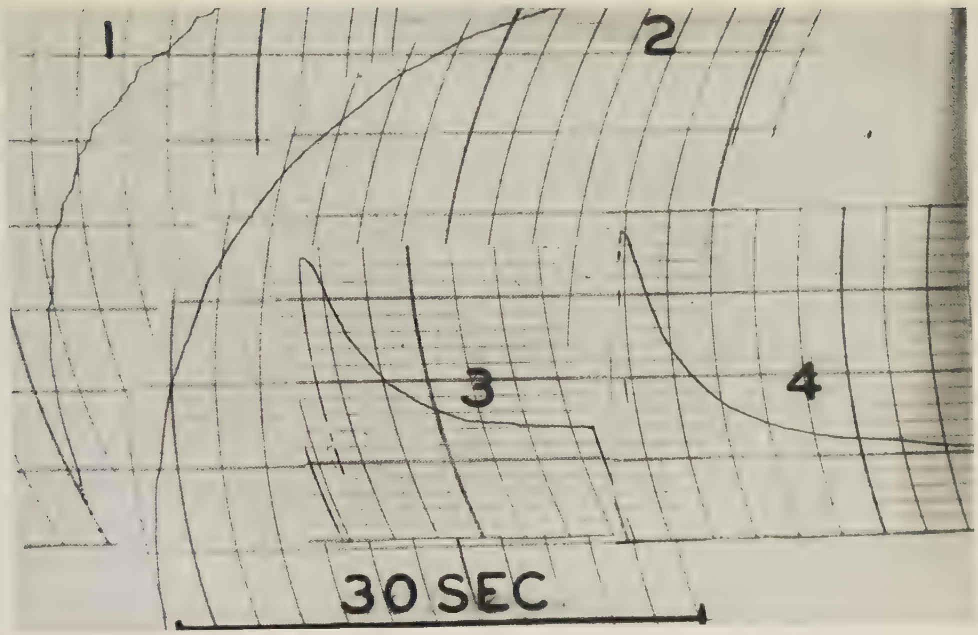


Fig. 7 The lack of effect of agglutination on the rate of swelling and shrinking of rabbit erythrocytes in ethylene glycol solutions. 1, Control cells swelling in 0.3 M ethylene glycol in R.L. 2, Cells agglutinated by $2.4 \times 10^{-2}\%$ tannic acid swelling in the same solution. 3,4, Control and agglutinated cells shrinking in $1.75 \times$ R.L. showing no effect of agglutination.

The observations mentioned above make it possible to state that changes in the degree of agglutination during the course of the measurements in the densimeter could not account for the observed differences in the light changes as recorded in the densimeter between the control and experimental cells. There is, however, another possibility. It might be assumed that agglutinated cells would have less surface area available for the diffusion of molecules into and out of them and this could explain the decrease in permeability observed with the experimental cells. If it is further assumed that an increase in the concentration of tannic acid would make less of the surface area available for diffusion, this could explain the observed increase in effect with increased concentrations of tannic acid. However, this could not explain the increased effect with larger penetrating molecules unless one were to make the unlikely assumption that that part of the surface containing the larger "pores" was being preferentially agglutinated. A much more plausible explanation would be that tannic acid was effectively decreasing the size of the "pores." This would be relatively more effective in slowing down the rate of penetration of the large molecules, which could enter originally through only a small number of "pores," than in slowing down the rate of penetration of the small molecules which could pass through many "pores."

As a final test of the effect of agglutination *per se* on the rate of entrance of erythritol, the experiment shown in figure 8 was performed. Curve 1 shows the rate of hemolysis of human erythrocytes (type A) in 0.2 M erythritol. Curve 2 shows the lack of effect of agglutination of these cells with anti-A serum on the rate of hemolysis in erythritol. These cells were sufficiently agglutinated so that they formed a solid mass after standing in the refrigerator over night. This mass was broken up before pipetting the cells into the densimeter. Curve 3 shows the increase in the time for hemolysis in the presence of $2.5 \times 10^{-3}\%$ tannic acid and curve 4, the effect of $2.5 \times 10^{-2}\%$ tannic acid. After 6 hours in the presence of

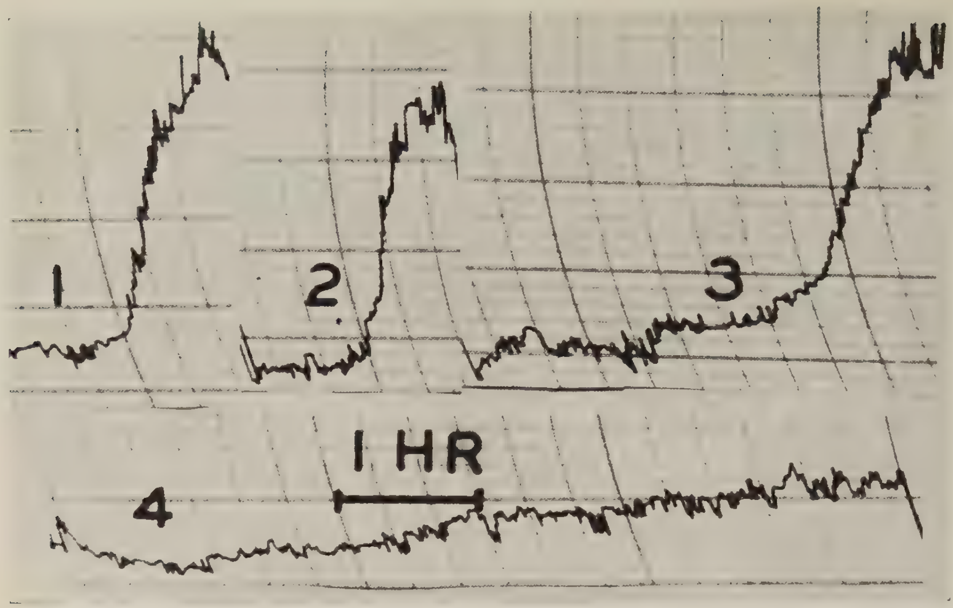


Fig. 8 A comparison of the effect of agglutination and of tannic acid on the rate of hemolysis of human erythrocytes in 0.2 M erythritol.

1. 10 cm^3 0.2 M erythritol plus 0.1 cm^3 cells.
2. 10 cm^3 0.2 M erythritol plus 0.1 cm^3 cells agglutinated with anti-serum.
3. 9.9 cm^3 0.2 M erythritol plus 0.1 cm^3 0.2 M erythritol containing 0.25 mg tannic acid plus 0.1 cm^3 cells.
4. 9 cm^3 0.2 M erythritol plus 1 cm^3 0.2 M erythritol containing 2.5 mg tannic acid plus 0.1 cm^3 cells.

TABLE 7

Per cent hemolysis and pH of suspensions of rabbit erythrocytes at various stages during an experiment

Supernatant fluid obtained by centrifugation following:	% hemolysis		pH	
	Control	Experimental	Control	Experimental
Initial swelling in M/6 NH_4Cl in R.L.-PO_4	5 5	6 4	7.4 7.5	7.4 7.4
Initial shrinking in 1.5 × R.L.	5 4	4 6	7.9 8.0	7.7 7.7
Second swelling in M/6 NH_4Cl in R.L.-PO_4	4 3	3 4	7.4 7.5	7.5 7.4
Second shrinking in 1.5 × R.L.	4 8	3 5	8.0 7.7	8.1 7.7
Initial swelling in 0.3 M glycerol in R.L.	3 2	4 4	7.9 7.9	7.8 7.7
Initial shrinking in 1.5 × R.L.	4	6	7.9	8.1
Second swelling in 0.3 M glycerol in R.L.	2	4	8.1	8.1
Second shrinking in 1.5 × R.L.	1	2	7.4	8.1
Third swelling in 0.3 M glycerol in R.L.	2	2	8.0	7.8
Third shrinking in 1.5 × R.L.	2	2	8.2	8.1

this latter concentration of tannic acid the cells were so completely tanned that they could not be hemolyzed even in water.

Another factor which might make the interpretation of the data difficult would be if considerable hemolysis occurred during the measurements in the densimeter. Data from typical experiments are shown in table 7. It can be seen that in every case there is a small amount of hemolysis but that there is no consistent difference between control and experimental cells. The last two columns show that there are no consistent differences in pH between control and experimental cells.

In a few experiments in which high concentrations of tannic acid were used (1.8×10^{-2}–1.0×10^{-1}) there was considerably more hemolysis particularly during the initial shrinking in 1.75 × R.L. which suggests that the experimental cells were more fragile (frail—cf. Hunter, '53) than the controls. Even in these experiments, however, differences in rate of swelling of control and experimental cells could be observed. In these solutions (swelling) there was little difference in the amount of hemolysis in the control and experimental solutions.

SUMMARY

In spite of other possible explanations, the observations can best be interpreted by assuming that tannic acid affects the membranes of erythrocytes of human, rabbit and chicken in such a way that the rate of entrance and exit of non-electrolytes such as ethylene glycol, glycerol and erythritol is decreased. In general, higher concentrations of tannic acid are required to decrease the rate of movement of non-electrolytes than of anions. The effect is more apparent when the rate of movement of larger molecules is studied and higher concentrations of tannic acid have more effect than lower concentrations. These changes are easily partially reversed. It is interesting to postulate that in the presence of tannic acid the structure of the proteins in the membrane is altered in such a way that the effective size of the "pores" in the membrane is reversibly decreased.

CONCLUSIONS

1. The permeability of human erythrocytes to anions, glycerol and erythritol is reversibly decreased by tannic acid.
2. The permeability of rabbit erythrocytes to anions, ethylene glycol, glycerol and erythritol is reversibly decreased by tannic acid.
3. The permeability of chicken erythrocytes to ethylene glycol and glycerol is reversibly decreased by tannic acid.
4. The concentration of tannic acid necessary to change the permeability of these cells to non-electrolytes is higher than that necessary to change the permeability to anions.
5. In general, higher concentrations of tannic acid decrease the rate of movement of non-electrolytes more than do lower concentrations.
6. The decrease in permeability in the presence of tannic acid is more easily demonstrated using large molecules than using small ones.
7. Although in the present experiments there was agglutination of the cells by the tannic acid, it is believed that this cannot explain the observed changes.
8. In higher concentrations, tannic acid increases the fragility of erythrocytes.

LITERATURE CITED

Boyden, S. V. 1951 The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by anti-protein sera. J. Exp. Med., *93:* 107–120.

Edelberg, R. 1952 The action of tannic acid on the erythrocyte membrane. J. Cell. and Comp. Physiol., *40:* 529–548.

——— 1953 The action of tannic acid on model systems formed of constituents of the erythrocyte membrane. Ibid., *41:* 37–50.

Ellis, S. C., and K. G. A. Pankhurst 1954 The interaction of tanning materials with collagen monolayers. Disc. of the Faraday Soc., *17:* 170–179.

Feinberg, R. J., and J. A. Flick 1954 Simple *in vitro* method for measuring antibodies in serum of hay fever patients. Fed. Proc., *13:* 492–493.

Gustavson, K. H. 1949 Some protein-chemical aspects of tanning process. Adv. in Prot. Chem., *5:* 353–421.

——— 1954 Some aspects of the reaction of basic chromium salts with hide proteins. Disc. of the Faraday Soc., *17:* 185–195.

——— 1956 The Chemistry of Tanning Process. Academic Press, Inc., New York, N. Y.

Hoffman, J. F. 1952 The action of uranyl salts on the erythrocyte surface. Biol. Bull., *103:* 303–304.

Hoffman, J. F., R. H. Collier and M. J. Deitch 1955 Semi-quantitation of the rate of shape transformation (of constant volume) of human erythrocytes. J. Cell. and Comp. Physiol., *46:* 355.

Hunter, F. R. 1953 An analysis of the photoelectric method for studying osmotic changes in chicken erythrocytes. Ibid., *41:* 387–406.

Hunter, F. R., D. Chalfin, F. J. Finamore and M. L. Sweetland 1956 Sodium and potassium exchange in chicken erythrocytes. Ibid., *47:* 37–54.

Jacobs, M. H., D. R. Stewart and M. K. Butler 1943 Some effects of tannic acid on the cell surface. Am. J. Med. Sci., *205:* 154.

Jacobs, M. H., and D. R. Stewart Unpublished data.

Mawe, R. C. 1956 The diffusion of glucose into the human red cell. J. Cell. and Comp. Physiol., *47:* 177–214.

Parpart, A. K., and R. Ballentine 1943 Hematocrit determination of relative cell volume. Science, *98:* 545.

Schulman, J. H., and M. Z. Dogan 1954 Tanning of fatty acid, amino acid and protein monolayers by metal ions. Disc. of the Faraday Soc., *17:* 158–170.

Visual Responses in Crayfish

I. RECORDING SHOCK RESPONSES TO LIGHT WITH REMOTE ELECTRODES[1],[2]

GEORGE CAMOUGIS[3]
Biological Laboratories, Harvard University, Cambridge, Massachusetts

As part of an extensive investigation of visual responses in crayfish of the species *Orconectes virilis* (Hagen), it was noted that shock responses occurred when the animals were suddenly illuminated. The term "shock response" is adopted from Wolf and Zerrahn-Wolf ('36) who used these words in describing the backward movement reaction of sunfish as the stimulus light was turned on. Crayfish respond by sudden changes in body tone or by movements of appendages. Since the study of visual responses included the timing of electrical responses to illumination at various levels of the stimulus-response chain, it became desirable to have precise measurements of the latent periods of the shock responses. Earlier studies on reactions of aquatic arthropods to light (Stehr, '31) were restricted by limitations in the recording method. In the present study it was possible to make accurate recordings of shock responses to light from freely moving crayfish by using remote electrodes.

MATERIALS AND METHOD

Recording electrodes were made of 12-cm lengths of 8-gauge (0.128 in. diam.) copper wire. One end was flattened for a length of about 2.5 cm for greater area. The electrodes were insulated with several layers of quick-drying nail polish except for the flattened, recording ends and about 2 cm of the opposite ends left bare for electrical contact. The experimental animal was placed in a glass crystallizing dish about 19 cm in diameter. One liter of distilled water was added, making the depth of the water about 4.5 cm. This depth of water allowed a large crayfish to move about freely. The electrodes were clamped on a rod and lowered perpendicularly into the water so that the recording ends were completely submerged but not in contact with the dish. Interelectrode distance was about 14 cm. The upper ends of the electrodes were then connected to a preamplifier. A cylinder 15 inches in diameter and 8 inches high was made of flexible frosted acetate sheeting cemented to a Lucite frame. The inverted cylinder was mounted over the crystallizing dish and electrodes (fig. 1). A large inverted cone section was suspended around the cylinder. Two lamp sockets were mounted above the cone and supplied with 110 v. D.C. Tungsten filament lamps of various wattages were used as stimulus lamps. The inside of the cone was painted white to reflect the light from the stimulus lamps overhead. The reflected light was further diffused by the frosted acetate cylinder The light was not uniformly bright around the entire circumference. However, the variation was not very great. Light intensity was measured with a Macbeth illuminometer placed inside the acetate cylinder and aimed horizontally at the illuminated cone. Table 1 shows the average luminance values in millilamberts (mL) for the light entering the cylinder when various light bulbs were used. The stimulating assembly, animal chamber, recording electrodes and a Grass P4 A.C. preamplifier were placed inside a shielded cage. The cage was closed and the animal allowed to become dark adapted for

---

[1] This paper comprised part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Harvard University.

[2] This investigation was supported in part by a fellowship, MF–7455, National Institute of Mental Health, Public Health Service.

[3] Present address: Department of Biology, Clark University, Worcester, Massachusetts.

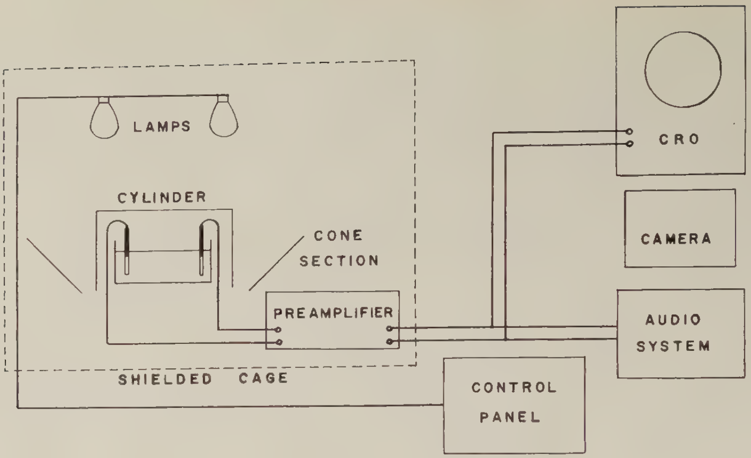


Fig. 1 Diagram of apparatus.

20–30 minutes. The output of the preamplifier was fed into a Dumont 322 cathode ray oscilloscope (CRO). An audio system was used for monitoring. Records were made with a Grass kymograph camera. The stimulus lights were operated from a switch mounted on the control panel. The switching artifact that appeared on the CRO when the light was turned on was used as a marker for onset on illumination (see oscillograms). A photocell was later used to measure accurate corrections for the lag time between switching and onset of stimulus illumination. All experiments were carried out at room temperature which varied from about 24 to 30°C.

TABLE 1

Luminance values of the stimulus lights

Wattage of bulbs	Luminance
	millilamberts
7.5	1.7
15	5.3
25	10.8
40	15.6
60	31.1
100	51.8
75 (Flood lamps)	84.6
150	121.0

RESULTS

When the animal placed in the dish was quiet, the baseline of the CRO was steady. Then, as the animal moved, deflections in the order of 50–100 μv could be readily detected. When the animal was very close to or in actual contact with the electrodes, the potentials could reach an amplitude of several hundred microvolts. Extensive slow oscillations of the surface of the water tended to generate corresponding, slow electrical deflections of the CRO. Usually, however, surface disturbance was minimal.

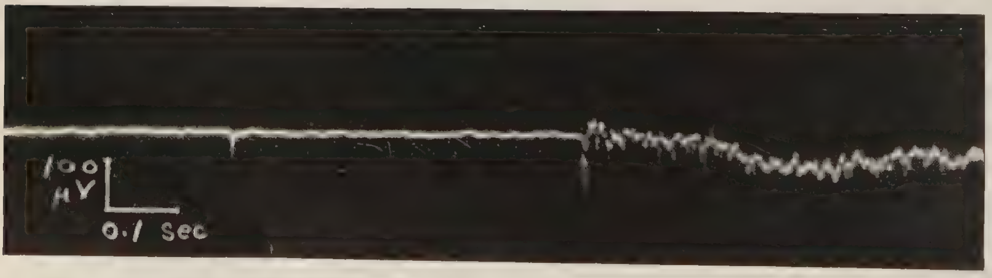


Fig. 2 Oscillogram of shock response showing sustained response. The arrow indicates the onset of response. The switching artifact has been slightly retouched for photographic reproduction.

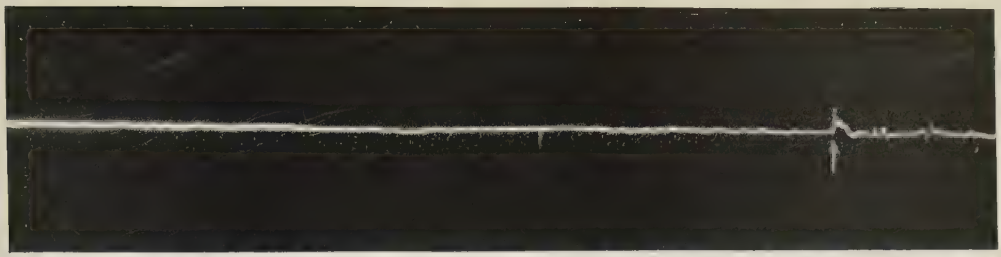


Fig. 3 The shock response consists of a vigorous initial response followed by relatively low activity. Magnitudes of time and voltage scales are the same as in figure 2.

TABLE 2

Data on shock responses

Luminance	Number of tests	Number of responses	Per cent responses	Average resp. time
mL				*sec.*
1.7	11	4	36	1.69
5.3	17	8	47	0.91
10.8	14	8	57	0.38
15.6	7	3	43	0.23
31.1	9	3	33	0.26
51.8	5	4	80	0.20
84.6	8	6	75	0.26
121.0	4	3	75	0.22
Totals	75	39	52	

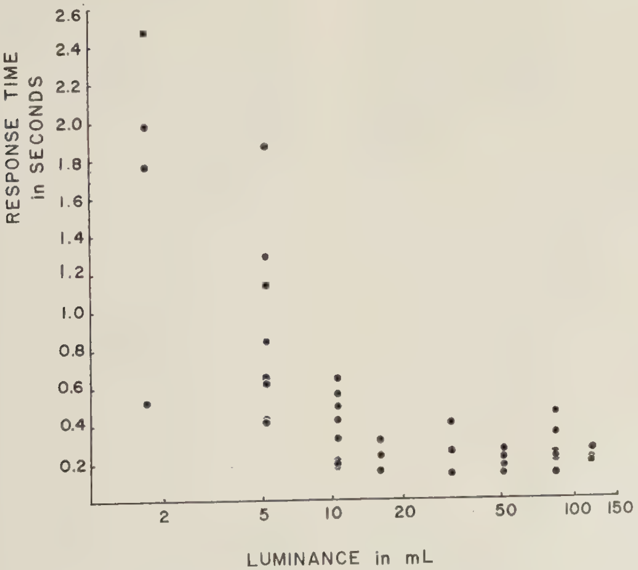


Fig. 4 Graph of shock responses.

After the animal was placed in the dish it moved about for a while, the activity being recorded very easily on the CRO and audio monitor. Almost all animals then became quiescent within a few minutes, perhaps moving only briefly a few times during the period of dark adaptation. When the light was turned on, the animals usually showed a shock response, characterized by the sudden postural change described earlier. This was especially noticeable on the audio system. The animals would sometimes show prolonged activity (fig. 2) or almost no continued activity (fig. 3) after the initial, vigorous response. In some cases the animal was not perfectly still before the stimulus and the baseline was therefore not absolutely flat. In these cases it was often difficult to determine whether there had been a definite response. Only clear cut deflections above baseline level were considered as definite responses. Table 2 shows the data on shock responses. It is to be noted that only about one-half of the 75 stimulus situations elicited a shock response. The more intense lights were generally more effective in eliciting a response. The 39 definite responses were plotted to show graphically how response time varied as a function of luminance (fig. 4).

DISCUSSION

Evaluation of method. The potentials recorded from the freely moving crayfish were mainly muscle action potentials. They resemble the monophasic muscle action potentials recorded from crustacean muscle by others (DuBuy, '35; Wiersma and Wright, '47). By watching the animal, it was readily apparent that the potentials corresponded to movements of the body and appendages. Individual antennal movements could be recorded if the other appendages were still. Movements can be recorded from an animal in tap water but the potentials are attenuated because of the comparatively lower resistance pathway between the electrodes caused by the greater number of ions in tap water as compared to distilled water. When animals are placed in very weak saline solutions, the signals are almost shorted out completely. Practically no potentials can be detected unless there is actual contact between electrode and animal. There is no concern over the problem of the polarization of the bare metal electrodes provided that fast, phasic transients without appreciable D.C. components are recorded. This method appears to be a suitable way of getting information on the movements of fresh water animals. The author has used it to record movements from fish, frogs, and aquatic insects. Essentially the same principles underlie the method used by Kleerekoper and Sibakin ('56) in their investigations of electrical potentials from sea lampreys. The method has the obvious advantage of not requiring the attachment of electrodes and wires to the bioelectrical generator. At the same time there is a limitation in the ability to localize the electrically active site. On the whole, however, the method of remote electrodes is a useful one in recording qualitatively and to a lesser degree quantitatively a variety of bioelectrical events in fresh water media.

Experimental results. Motor responses to sudden changes in illumination have been reported in clams (Hecht, '19), in barnacles (von Buddenbrock, '30), in various aquatic arthropods (Stehr, '31), and in sunfish (Wolf and Zerrahn-Wolf, '36). The clams respond to light by sudden contraction of their siphons. The responses of the barnacles consisted of a withdrawal reflex as a sudden shadow interrupted the incident light. The responses appeared to be reflexive in the Crustacea and Hemiptera tested by Stehr and consisted mainly of swimming movements. The shock responses of the sunfish consisted of the backward movement reaction described earlier. All these responses appear to be generally related and are probably concerned with protection and escape.

Table 2 indicates that two things are involved in these responses. These are the average response times and the percentages of responses for the various light intensities. An examination of figure 4 raises the question as to whether an average response time has any valid meaning in the tests involving the lower luminance values. It may not because the ranges are very large and the percentages of responses are low. However, there is a definite inverse relation between light intensity and response time at the lower luminance values. If the average response times are used, the Bunsen-Roscoe reciprocity relation does not apply to any part of the light intensity range as it does with the photochemical events underlying the responses of *Mya* (Hecht, '19, '21). Stehr ('31) also found inverse relations between light intensity and reaction time in the 6 species of aquatic arthropods that he tested. He also concluded that the data did not follow any formula such as the Bunsen-Roscoe Law. Critical examination would undoubtedly show that the reciprocity relation holds for the primary photochemical event underlying photoreception which then leads to subsequent response after a latent period. But to expect the reciprocity law to apply to total response times of the crayfish and the aquatic insects is unreasonable because of so many factors that may influence the central nervous system which must mediate the responses. For example, the shortest response time (0.52 sec.) on the graph for 1.7 mL was produced by an animal that was hyperactive, as evidenced by constant movements during dark adaptation. Such an animal could require less external stimulation to elicit a response. Stehr ('31) also found large variabilities in the response times of all the species that he tested. He found *Cambarus* to be very variable and stated that it was often difficult to judge when an animal responded. He got no responses from one-third of the tests at light intensities comparable to the lower values used in these experiments. This agrees with the present data which show the greatest ranges in response times and low percentages of response at the lower intensities.

After the initial period of inverse relation between light intensity and response time, the response times approach the luminance axis of the graph asymptotically. The same is true with the responses to visual stimuli in *Mya* (Hecht, '19), *Balanus* (von Buddenbrock, '30), and man (Woodworth and Schlosberg, '54). Figure 4 shows that about 15 mL is the critical intensity above which the response times show comparatively little variation. The range of all responses above 15 mL is 0.13 to 0.46 seconds. The average response times are closely grouped from 0.20 to 0.26 seconds (table 2). Most of the responses at the higher luminance values are undoubtedly reflexive startle responses with perhaps minimal response times involved. One would expect response times to reflect the summed delays of the sensory, coordinating, and response mechanisms. There is great variability in reaction times not only in crayfish but also in other animals. For example, the responses of moths to ultrasonic sound take 75 to 262 msec. (av. 139 msec.); roaches respond to air blasts in 28 to 90 msec. (av. 54 msec.); bees may respond after 70 msec or take as long as 300 to 400 msec. in the tarsal flight reflex (Roeder, '59). The reaction times of humans to visual stimuli range from 130 to over 200 msec. and these vary with the psychological state of the subject (Woodworth and Schlosberg, '54). Perhaps the reaction times are not merely the arithmetic sum of the sequence of all neural events in the stimulus-response chain. An analysis of the constituent neural events underlying the startle response of the roach resulted in conclusions suggesting that different states of central adaptation may modify the response time (Roeder, '59). A similar analysis was made at various levels of the stimulus-response chain in crayfish and it was found that the central nervous system had a labile integrative role in mediating visual responses (Camougis, in preparation). With the degree of neural complexity involved in the reactions discussed above, it is not surprising that reaction times cannot be predicted merely by knowing the summation of the times required for the neural events underlying the reactions. After all, the supersedure of automaticity is the intriguing attainment of the more advanced nervous systems.

ACKNOWLEDGMENTS

I wish to thank Professor J. H. Welsh for his advice during the course of this investigation. I also wish to thank Dr. W. S. von Arx for discussing the recording of electrical phenomena in aquatic media.

LITERATURE CITED

DuBuy, H. G. 1935 The electrical phenomena of the crustacean nerve-muscle system. Am. J. Physiol., *114:* 224–234.

Hecht, S. 1919 The nature of the latent period in the photic response of *Mya arenaria.* J. Gen. Physiol., *1:* 657–666.

——— 1921 Time and intensity in photosensory stimulation. Ibid., *3:* 367–373.

Kleerekoper, H., and K. Sibakin 1956 An investigation of the electrical "spike" potentials by the sea lamprey (*Petromyzon marinus*) in the water surrounding the head region. J. Fish. Res. Bd. Canada, *13:* 375–383.

Roeder, K. D. 1959 A physiological approach to the relation between prey and predator. Smithson. Misc. Coll., *137:* 287–306.

Stehr, W. C. 1931 The activating influence of light upon certain aquatic arthropods. J. Exp. Zool., *59:* 297–335.

von Buddenbrock, W. 1930 Untersuchungen über den Schattenreflex. Zeit. Vergl. Physiol., *13:* 164–213.

Wiersma, C. A. G., and E. B. Wright 1947 The nature of the action potentials of crustacean muscles. J. Exp. Biol., *23:* 205–212.

Wolf, E., and G. Zerrahn-Wolf 1936 Threshold intensity of illumination and flicker frequency for the eye of the sunfish. J. Gen. Physiol., *19:* 495–502.

Woodworth, R. S., and H. Scholsberg 1954 Experimental Psychology, revised ed. Holt, New York.

Spontaneous Electrical Activity in the Crayfish Central Nervous System[1]

JOSEPH K. HICHAR[2]
Harvard University, Cambridge, Massachusetts

Crayfish central nervous system ganglia have proved to be suitable preparations for the study of certain nervous phenomena. Since the discovery of spontaneous electrical activity in these ganglia by Prosser ('34), our knowledge of synaptic transmission and drug actions has increased considerably, but in spite of the substantial amount of work done with this preparation some parameters which might influence its spontaneous activity have not been investigated. Also, with the substantial background of studies of effects of drugs on this preparation, it was decided that investigations of some drugs which have more recently gained neurophysiological significance would be desirable. Those drugs and parameters which were studied and are reported here follow.

1. *Effects of serotonin and related drugs.* Evidence is mounting implicating serotonin (5-HT) as a neurohumor involved in synaptic transmission (Florey and Florey, '54; Welsh, '57). Its transmitter role in Crustacea was first proposed by Florey and Florey ('53, '54) in studies involving extracts of nervous tissue which contained an active principle which reacted similarly to 5-HT. It was found to excite crustacean hearts (Florey and Florey, '54; Welsh, '57) and proprioceptors in legs and abdomens (Florey, '54). It was decided therefore to study the effects of 5-HT on the spontaneous electrical activity of the abdominal ganglia of crayfish.

Also studied in this connection were the effects of lysergic acid diethylamide (LSD), a relative of 5-HT which is both a mimetic and an antagonist in invertebrates (Welsh, '54, '57) and vertebrates (Woolley, '55). Iproniazid, which was found to be an anti-amine oxidase (Zeller et al., '52; Shore et al., '57; Spector et al., '58), was also investigated to determine a possible connection with 5-HT.

2. *Effects of gamma-aminobutyric acid, beta-alanine and picrotoxin.* Gamma-aminobutyric acid (GABA) imitates the action of inhibitory neurones in crayfish (Edwards and Kuffler, '57) and upon injection into the animal results in flaccidity (Bazemore et al., '56). Picrotoxin antagonizes this effect (Bazemore et al., '56; Florey, '57b; van der Kloot et al., '58) although Schallek and Wiersma ('48) observed no effect on synaptic transmission using 2.5×10^{-4} M picrotoxin. Bazemore et al. ('56) reported beta-alanine to have effects similar to those of GABA on the crayfish stretch receptor but to be 1/20 as active. The effects of GABA were studied along with picrotoxin and beta-alanine to determine the possible inhibitory neurohumoral role of GABA.

3. *Effects of dimethylaminoethanol, possible precursor of acetylcholine.* Pfeiffer et al. ('57) have presented evidence implicating dimethylaminoethanol (DMAE) as a precursor of acetylcholine (ACh). They found that in man 10 to 20 mg per day caused mild central nervous stimulation, whereas 10 gm per day of choline, the supposed precursor, resulted in no stimulating action whatever. If it is a precursor of ACh in the crayfish, one might detect increased activity upon the application of DMAE if the system under study is a cholinergic one as appears to be the case from the indirect evidence. This was

---

[1] This paper is extraced from the Ph.D. thesis, *Spontaneous electrical activity in the crayfish central nervous system*, Harvard University, 1958 and was supported in part by research fellowship BF-7367 from the National Institute of Neurological Diseases and Blindness, Public Health Service.

[2] Present address: Department of Biology, Parsons College, Fairfield, Iowa.

investigated together with a restudy of ACh, its mimetics and cholinesterases.

4. *Variation of ACh content in nervous system and correlation with spontaneous activity.* It had been reported that variations in the ACh content occurred in the crayfish nervous system, there being three times more ACh present in the spring than in the fall (Smith, '39). This is an extremely interesting observation and demands attention if ACh has a function in the crayfish similar to that in the vertebrates. Colhoun ('58) recently found a correlation between nervous activity and the amount of ACh in the cockroach. Van der Kloot ('55) found spontaneous activity and cholinesterase (ChE) content in the *Cecropia* larval brain were closely correlated and that they varied with stages in the life cycle. The possibility of such a correlation between electrical activity and ACh content was studied here.

An additional attempt to tie in electrical activity with ACh content was made by correlating the ACh content as found by bioassays with the activity in nerve cords which had different levels of electrical activity.

5. *Other parameters.* As the size of crayfishes increases so do the nerve cords and ganglia increase, and presumably the fibers comprising the cords likewise increase. If this is so one might find proportionately more activity in the larger animals since more fibers would have large enough action potentials which would be felt above the noise level of the recording system. This possibility was investigated along with the effects of physical condition, sex, aggressiveness, season, laboratory temperature and length of time of recording.

METHODS

Electrical recordings of spontaneous activity were from the isolated 5th abdominal ganglion of the crayfish, *Orconectes virilis*. Modified ruling pen electrodes clamped the connectives on both sides of the ganglion which was held below the connectives by a glass hook, the whole assuming a "V"-shape. This minimized changes in levels of electrical activity due to the mechanical stimulation involved in traversing an interface between water and oil. A Stender dish containing van Harreveld's solution (VH) (van Harreveld, '36) with a layer of light mineral oil on top was placed on a variable-height stage, and the preparation immersed in it. When it was desired to record, the stage was lowered, thus moving the ganglion from the VH into the oil phase.

The electrical activity was led through the push-pull circuit of a pre-amplifier which was located in a shielded cage along with the preparation and fed simultaneously to a cathode-ray oscilloscope, a tape recorder, an audio-amplifier, and an electronic scaler. From the scaler the signals were fed through an integrator, a special circuit which was necessary to modify the time characteristics of the integrator, and finally into a graphic ammeter. Tape recordings were made so that counts of electrical activity at specific intervals in different animals could later be compared.

After mounting the preparation in the electrodes, the activity was recorded for one minute and the preparation then immersed in VH for at least ½ hour to allow the initial injury activity (immediate activity) to wane. It was found that after this period of time activity was stabilized for at least 10 hours. Since most experiments took considerably less time than this, it is reasonable to assume that the activity of the preparation did not fluctuate due to injury. After the ½-hour rest period, the preparation was raised into the oil phase and the activity recorded for one minute. This was considered to be the control for the subsequent recording which took place immediately after immersing the ganglion in the test solution. This recording of activity was normally conducted for about three minutes after which the cord was rinsed in VH. After being in the rinse for about 5 minutes, the preparation was immersed in fresh VH and after at least 10 minutes the procedure was repeated.

In the studies of animal size, physical condition and variation of ACh content on spontaneous activity, similar sections of tape-recorded activity were compared. These sections were of normal activity, which was defined as that activity following the first VH rinse after the immediate activity. The base-line of noise level in each preparation was determined by making the preparation electrically silent by immersion in 3 M KCl upon completion of the experiment.

The studies concerned with the time of year and its effect on spontaneous activity were conducted in two ways. In the first, crayfish were trapped once a month throughout the year and brought back to the laboratory as soon as possible where the normal electrical activity of the 5th abdominal ganglion was recorded. In the second approach, animals were maintained in aquaria in the laboratory and the month they were used for experimentation was recorded along with their levels of normal activity.

The bioassays for total ACh content of the ganglia and nerve cords were conducted on the heart of *Venus mercenaria.* The clam heart preparation was made and set up according to the method of Welsh and Taub ('48). After arranging the heart in the chamber, a 10^{-7} M LSD solution was placed in the chamber and the heart contractions allowed to reach their maximum amplitude. Meanwhile the electrical activity of the nerve cords or 5th abdominal ganglia was recorded. The cords were then weighed and placed in 1 or 2 cm^3 of hot VH to destroy the ChE present. The tissue was kept at about 100°C for 10 minutes and then homogenized. Dilutions were then made of the homogenate and these were applied to the *Venus* heart and the depression of amplitude noted. These depressions were then matched with depressions caused by the addition of known concentrations of ACh.

Experiments were conducted at room temperature which normally varied from 24 to 27°C. The pH of the VH normally was close to 7.0, ranging from 6.5 to 7.8. In those cases where test substances increased the pH above this range, the solution was brought to 7.0 by the addition of HCl.

RESULTS

Using the normal activity in the last 100 experiments in this series it was found that the average number of impulses recorded from the ganglion was 57/sec. with a minimum of zero and a maximum of 447/sec. (Hichar, '60a). Size of the animals, as well as their sex, time of the year, stress, aggressiveness, length of time from which recorded and laboratory temperature appeared not to have a significant influence on the frequency of firing. That is, the variation that normally is evident in spontaneous activity from animal to animal was sufficiently great that no effect of these factors could be detected. (Statistical details can be found in the thesis.)

Although Roeder ('41) reported steady levels of activity in 90% of his crayfish abdominal nerve cord preparations, it was found not infrequently that when the graphic ammeter chart speed was increased sufficiently, a rhythmicity of the spontaneous activity could be detected as illustrated in figure 1. The records in the figure show the levels of spontaneous activity for one minute in 4 different preparations. In A one can see rhythmical increases in spontaneous activity occurring at a rate of 8/min. In B the rhythmicity is half that or 4/min. In C it is 5/min., and in D, 2/min.

The effects of the various drugs tested are shown in table 1. The threshold and inhibitory concentrations and reversibility given were what most preparations showed. Occasionally a preparation would have a threshold up to 100 times that of the average; on the other hand, at times one would be completely unresponsive. In general, concentrations tested were no greater than 10^{-2} M.

The variation in levels of spontaneous activity of the 5th abdominal ganglion of the crayfish was recorded from 10 animals. The cords used in these experiments were then separated according to the amount of activity into three groups: (1) those with low activity, (2) those with high activity, and (3) those which were intermediate. Bioassays on the *Venus* heart revealed that it was not possible to distinguish different amounts of ACh in these three groups of nerve cords. The difference between the average counts of the highest and lowest groups was 5,300 action potentials/minute. Yet their levels of ACh were essentially the same, about 6 gamma/gm tissue, ranging from 4.97 to 8.5 gamma/gm (fig. 2A). Observations on 4 similar experiments gave comparable results.

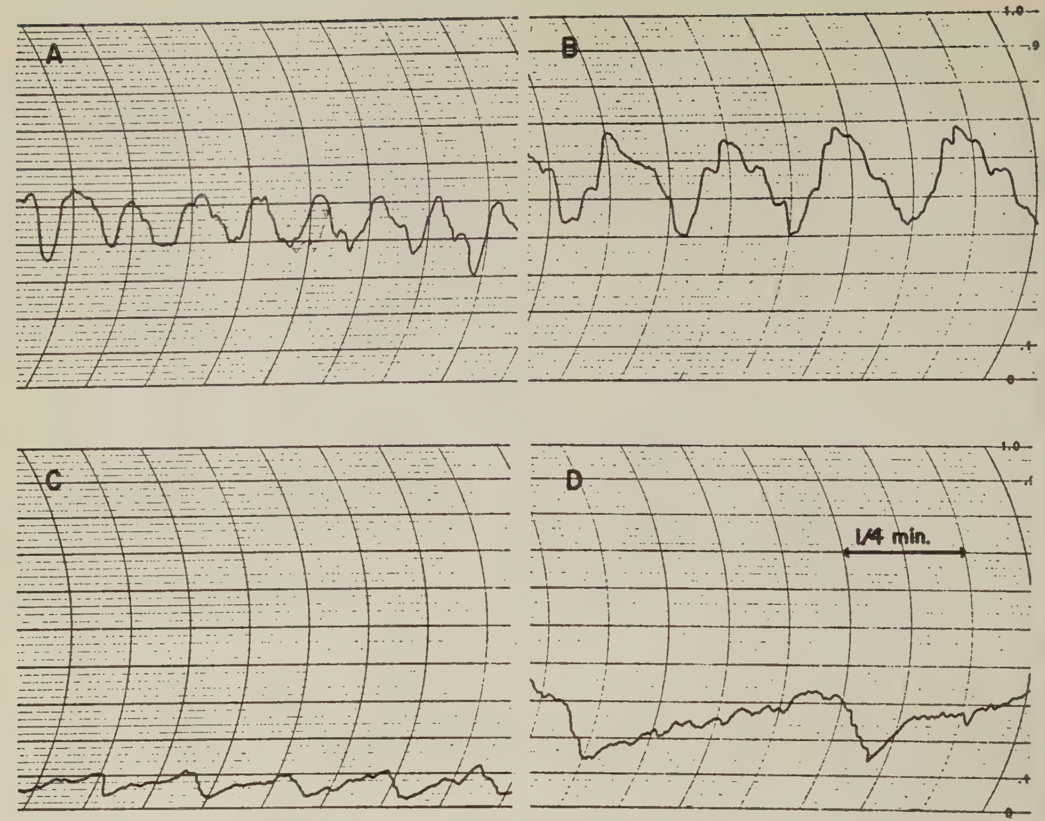


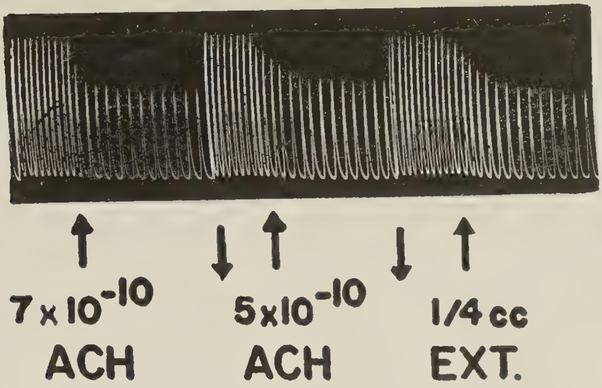
Fig. 1 Various rhythms of nervous activity superimposed on normal levels of spontaneous activity. Vertical lines delineate ⅛ minute intervals. A, 8/minute; B, 4/minute; C, 5/minute; D, 2/minute.

TABLE 1

Effects of selected substances on spontaneous activity

Substance	Threshold concentration	Inhibitory concentration	Reversibility
	M	*M*	
5-HT	10^{-2}	?	?
5-HT and Iproniazid	10^{-2}	?	?
LSD	10^{-5}	10^{-3}	No
GABA	?	?	?
Beta-alanine	10^{-4}	10^{-2}	No
Picrotoxin	10^{-8}	10^{-4}	No
DMAE	10^{-5}	?	No
ACh-Cl	10^{-3}	?	Yes
ACh-Br	10^{-3}	?	Yes
Nicotine	10^{-6}	10^{-4}	Yes
Pilocarpine	10^{-5}	?	Yes
Eserine	10^{-8}	10^{-3}	?
Prostigmine	10^{-4}	?	?

A. NORMAL CORDS



B. LOW ACTIVITY NERVE CORD

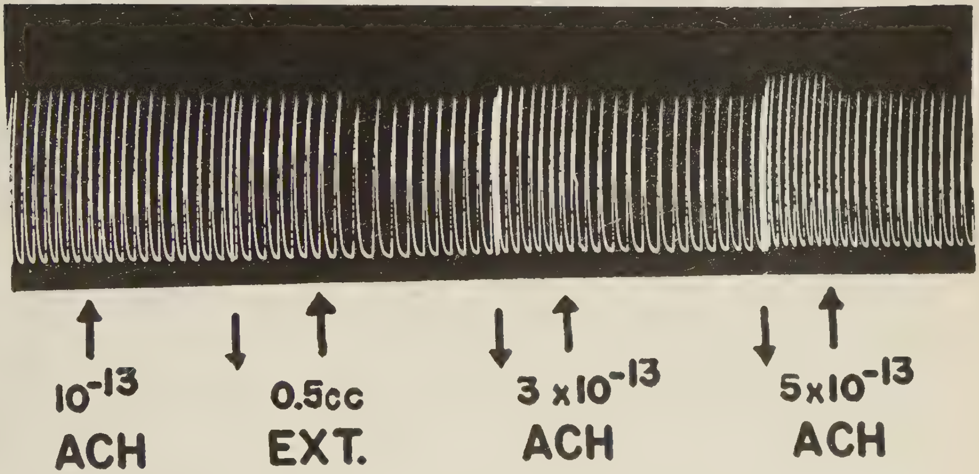


Fig. 2 Kymograph records of contractions of clam hearts used in bioassays for ACh. A, Nerve cord with "normal" level of spontaneous activity. B, Electrically "silent" nerve cord. Arrows pointing downward indicate periods of rinsing in sea water. Arrows pointing upward indicate additions of various concentrations of ACh or various amounts of nerve cord extract.

Bioassays for ACh in three other cords with little activity gave extremely interesting results. Bioassays were done: (1) on a cord which had very little activity at the start (fig. 3A), (2) on one which had rapidly decreasing activity (fig. 3B), and (3) on one in which activity decreased at a slower rate (fig. 3C). The amount of ACh assayed in the cords was 0.001 gamma/gm tissue in (1) 0.0007 gamma/gm tissue in (2), and 0.0016 gamma/gm in (3) as shown in figure 2B. Since nerve cords with normal activity were found to have an average of 6 gamma ACh/gm tis-

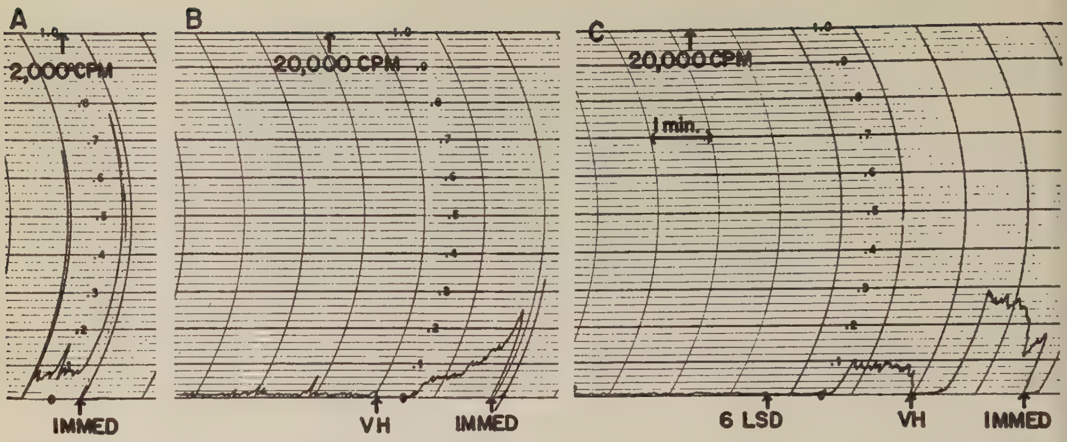


Fig. 3 Records of integrated spontaneous activity in three low activity or "silent" nerve cords. Records should be read from right to left. Full scale in A is 2,000 impulses/minute; in B and C, 20,000 impulses/minute. Vertical lines delineate one minute periods of recording. "Immed." shows activity recorded immediately after isolation of the ganglion; VH indicates rinsing in van Harreveld solution for 30 minutes; 6 LSD indicates activity recorded after immersion of the ganglion in 10^{-6} M LSD solution.

sue and these low activity cords had 0.001 gamma/gm on the average, there apparently was 6,000 times less ACh in those cords with little activity than in the cords with "normal" activity.

The electrical activity which was evident during the periods when it was recorded from these cords appears in figure 4. In figure 4A, one sees very little activity above the noise level. This activity averaged about 150 impulses/minute. In figure 4B the activity declined very rapidly during the immediate activity from a high point of about 5,000 counts/minute at the outset to about 200 impulses/minute one minute later. In figure 4C the activity at first increased to about 5,500 counts/minute, then dropped to 1,800/minute after 30 minutes in VH, and to about 100 impulses/minute one minute later. In "normal" cords containing 6 gamma ACh/gm it was not possible to distinguish different amounts of ACh in those where the rate of firing varied from an average of 13,700 impulses/minute to 19,000 with a mean of 15,000. In the low activity cords it appeared there was 6,000 times less ACh and their average rate of firing was 466 action potentials/minute (these cords varied from 108 to 930 counts per minute at the point when the bioassays were undertaken). It thus appears that with 30 times more electrical activity, normal cords had 6,000 times the ACh content of these three low activity cords.

DISCUSSION

Since no significant differences existed in the spontaneous activity of the isolated ganglia of animals differing in size, sex, physical condition, and aggressiveness and since time of year and temperature (within certain limits) did not significantly affect the electrical activity, it meant that all results obtained could be fairly compared with each other; that is, it was not necessary to take these factors into account in studying records of activity obtained.

The rhythmicity in the spontaneous activity always involved several cells. The rate varied and a correlation with behavioral or physiological phenomena was not possible. This rhythmicity is probably a reflection of an internal functional or chemical process associated with metabolism. The location of the cord in the body in very intimate contact with its environment (blood) as well as its sensitivity to chemical conditions prompts this suggestion.

The results with 5-HT were inconclusive. In two experiments it excited; in 6 it had no effect. After pre-treatment with iproniazid no change in spontaneous activ-

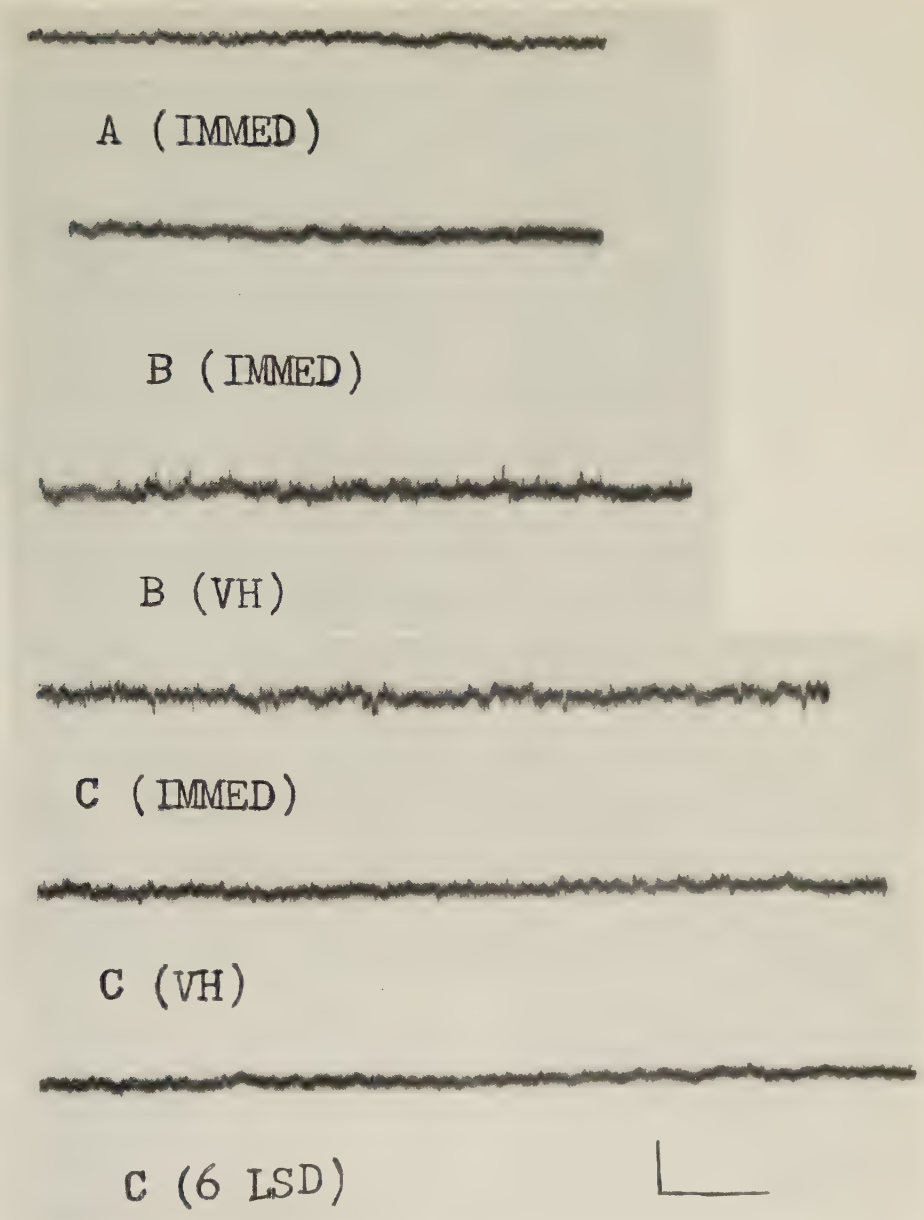


Fig. 4 Photographs of the electrical activity seen on the oscilloscope screen from the three "silent" preparations with low activity illustrated in figure 3. Calibrations: 20 μv., 100 msec. Other legends same as in figure 3.

ity was detected. Burgen and Kuffler ('57) observed that 5-HT inhibited the cardiac ganglion of Limulus, whereas Welsh ('57) reported that it excites the isolated heart of *Cancer* and Florey ('54) found that it increases the discharges of crustacean proprioreceptors. The lack of effect of 5-HT on spontaneous activity observed in the majority of experiments reported here does, however, attain additional significance by the recent observations of Welsh (personal communication) who has found no evidence of the presence of 5-HT in the lobster central nervous system. An indole amine, similar to 5-HT, may be present in the modified neurones of pericardial organs (Carlisle, '56) and be involved in neurohumoral activity, but this must be borne out by analytical determinations. The fact that two of the preparations responded at rather low concentrations (10^{-5} M) of LSD strengthens the suggestion that perhaps a substance similar to 5 HT is present and is involved in spontaneous activity.

After LSD treatment, individual fibers often began to fire repetitively. This suggests an influence on synaptic transmission since when this did occur fibers having nearly the same size potentials were involved. The implication that these fibers are physiologically connected in a communication system involving LSD or an LSD-like substance seems reasonable when one recalls that the general level of activity remained the same during these bursts.

It seems very unlikely that GABA would have a general neurohumoral role in the crayfish as suggested by Florey ('57a, '57b) since no effect was obtained here. Its central effects appear to be quite different from those at the periphery (Edwards and Kuffler, '57). Similarly, beta-alanine has different effects peripherally (Bazemore et al., '56) from those observed here. Perhaps GABA is a precursor of the inhibitory transmitter as proposed by Hayashi ('58), or perhaps it, or a similar substance, might be an inhibitory neurohumor at some peripheral synapses but not in the central nervous system.

The great excitatory effect of picrotoxin on spontaneous activity strengthens the view that it may compete with an inhibitory synaptic transmitter for a receptor site, (van der Kloot et al., '58) and thus enhance activity. This hypothesis appears particularly attractive since in some experiments picrotoxin seemed to affect only particular fibers. This possibility is being investigated using the technique of pulse height analysis (Hichar, '60a). Attempts to show GABA-picrotoxin antagonism in the central nervous system were unsuccessful (Hichar, '60b). This makes more unlikely a central inhibitory neurohumoral role for GABA.

Confirmation of the results of Roeder and Roeder ('39) on the spontaneous activity of cockroach cords and of Prosser ('40) on crayfish nerve cords and Maynard ('55) on lobster cardiac ganglia of the effects of ACh, appears again to indicate the impenetrability and instability of this ester. It should be noted, however, that slightly lower thresholds were found in the work reported here. That similar effects were obtained here and in cockroach nerve cords is interesting in view of the fact that the sheath of cockroach cords has been found to be a diffusional barrier for potassium and sodium ions and ACh (Twarog and Roeder, '56). However, in several experiments (although not with ACh) in which the cellular sheath of crayfish cords was removed, this investigator could detect no difference in the reaction toward applied substances whether the sheath was intact or removed. Very few experiments were done, making this inconclusive and worthy of repetition. The crayfish nerve cord preparation is, however, generally believed to be more sensitive than that from certain insects to some chemical changes in the environment and there is some evidence for this (Gaudin, '37; Welsh, unpublished data). The work reported here also supports this observation. At any rate, even after desheathing cockroach cords the concentration of ACh necessary to give definite effects was so large as to be outside the physiological range (Twarog and Roeder, '57). The fact remains that the lack of any appreciable effect of applied ACh in physiological ranges must be explained. An explanation which has a precedent in mammals is described by Eccles ('57). A presumed cholinergic junction of motor axon collaterals with Renshaw cells in the cat is not affected by ACh (or prostigmine). In this case Eccles states that the reason may be that these compounds do not penetrate through the blood-brain barrier. Further, the fact that ACh and prostigmine are quarternary ammonium compounds which penetrate membranes with difficulty helps to account for their ineffective action. It may be that for this reason no effect is detected with physiological concentrations of ACh.

The mimetics of ACh tested here, that is nicotine and pilocarpine, gave comparable results at slightly lower concentrations than those published by Roeder and Roeder ('39) and Prosser ('40). The anti-cholinesterases, eserine and prostigmine, caused excitation at substantially lower concentrations (about 1,000 times in the case of eserine) than reported by others. The fact that the effects of prostigmine closely resemble those of eserine except the whole being displaced toward higher concentrations also illustrates the difference between the diffusion through membranes of tertiary amines (eserine) and quaternary amines (prostigmine and ACh). That prostigmine is reported to have direct cholinergic action (Riker et al., '46) was not indicated in these experiments. All this indicates, together with the other evidence concerning the presence of ACh and AChE that a functioning cholinergic system occurs in the crayfish central nervous system.

DMAE was seen to give an effect very much like that of ACh but at slightly lower (10 times) concentrations. The greater effect would be expected since it, like eserine, is a tertiary amine and is known to get past the mammalian blood-brain barrier (Pfeiffer et al., '57) whereas ACh does not penetrate the blood-brain barrier (Eccles, '57). The enhancement of spontaneous activity by DMAE suggests that it may be involved in ACh synthesis. The evidence for such a role for DMAE in crayfish is certainly stronger than it is for choline since Ellis et al. ('42) found no effect with concentrations of up to 2% choline on crayfish neuromuscular transmission. It is possible that the synthesis of ACh proceeds along more than one pathway. DMAE may be involved in one of these, with choline perhaps involved in another.

The bioassays for ACh content in crayfish nerve cords were indeed interesting. Figures given in the literature vary a good deal. Jullien and Vincent ('38) found up to 20 gamma ACh/gm tissue in the ventral nerve cord of *Astacus*. Smith ('39) found 14 gamma/gm in the fall and 45.8 gamma/gm in the spring in the ganglia of *Cambarus* and 3.2 to 5 gamma/gm to 12.6 gamma/gm in nerve fibers in the fall and spring respectively. Schallek ('45) found 14.4 gamma total ACh/gm in the ventral nerve cord of *Cambarus*. Tobias et al. ('46) found 28 gamma free ACh/gm tissue in the thoracic nerve cord of *Cambarus*. I found an average of 6 gamma total ACh/gm tissue in normal nerve cords. The variations can be accounted for by differences in the methods of extraction and assay, species and time of year.

The tremendous difference between total ACh content of normal nerve cords and those of the relatively inactive cords (6,000 times difference) is difficult to explain. This is especially so when it is recalled that both these groups of animals appeared completely normal behaviorally. It may be that in some animals the operation involved in isolating the ganglion causes the immediate release of most of the ACh so that the supply is greatly depleted by the time the bioassays were made.

In several preparations which had a very low or silent level of activity at the start, this author found that after one hour the level of activity slowly increased at a fairly constant rate. This would indicate if ACh is the transmitter responsible that there is indeed synthesis of the ester. Synthesis of ACh has been reported by Walop ('50, '51) in *Carcinus* and Easton ('50) in crayfish nerve cords. Frontali ('58) recently reported synthesis of ACh in housefly heads and cited other work indicating similar syntheses in bee heads and blowfly heads. In view of these results, and the correlation between electrical activity and ACh content found here and similar results reported by Colhoun ('58) in cockroaches, and the correlation between cholinesterase and electrical activity found in *Cecropia* larvae by van der Kloot ('55) it appears that arthropod nerve cords do synthesize ACh.

The fact that no significant differences were evident in the levels of spontaneous activity recorded from freshly caught animals at different times of the year is quite understandable. It was seen that animals with 30 times more electrical activity also had 6,000 times more ACh. Smith ('39) showed that the ACh content varied from 45.8 gamma/gm in spring to 14 gamma/gm in fall. Therefore, these studies attempted to distinguish a difference in electrical activity between one group of nerve cords having three times the ACh content of another. If one uses this figure, it appears that the attempt was made to show a difference in electrical activity in nerve cords amounting to 0.015 of other nerve cords. It is very unlikely, in view of the normal range of spontaneous activity, that differences in electrical activity due to seasonal variations in ACh content could be shown in this way.

SUMMARY AND CONCLUSIONS

A study of the spontaneous electrical activity of the 5th abdominal ganglion of the crayfish revealed the following:

1. The average rate of action potentials generated in this ganglion is 57/sec. with extremes of 0 and 447/sec.
2. The level of spontaneous activity in different animals varied sufficiently to indicate no significant differences in levels of spontaneous activity in animals of different size, sex, and aggressiveness, and the time of year, stress, length of time from which recorded and laboratory temperature.
3. Rhythmical cycles of increased activity which varied from 2/min. to 12/min. sometimes occurred superimposed on the normal level of activity.
4. Results of other workers using ACh and related compounds were confirmed. DMAE gave effects resembling those of ACh, which indicates a possible role as precursor for the latter substance.
5. Serotonin was found to be inactive at physiological concentrations in the majority of experiments. LSD had a threshold of 10^{-5} M, which lends credence to the possibility that an indole amine other than serotonin may be involved in spontaneous activity.
6. GABA had no observable effect on spontaneous activity. Beta-alanine and picrotoxin both caused excitation, the threshold of the former was 10^{-4} M and that of the latter was 10^{-8} M. No picrotoxin-GABA antagonism was demonstrable.
7. ACh content of abdominal nerve cords of completely normal-appearing animals was found to vary by as much as 6,000 times. Most cords had 6 gamma ACh/gm tissue but three cords, also from "normal" animals had 0.001 gamma/gm tissue. Electrical activity was found to vary by as much as 30 times. A correlation was evident between ACh content and electrical activity, which implies a cholinergic neurohumoral system.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the generous help and encouragement given me by Prof. John H. Welsh during the course of these investigations.

LITERATURE CITED

Bazemore, A., K. A. C. Elliott and E. Florey 1956 Factor I and GABA. Nature, *178:* 1052–1053.

Burgen, A. S. V., and S. W. Kuffler 1957 The inhibition of the cardiac ganglion of *Limulus polyphemus* by 5-HT. Biol. Bull., *113:* 336.

Carlisle, D. B. 1956 An indole-alkylamine regulating heart-beat in Crustacea. Biochem. J., *63:* 32 pp.

Colhoun, E. H. 1958 Tetraethyl pyrophosphate and ACh in *Periplaneta americana.* Science, *127:* 25.

Easton, D. M. 1950 Synthesis of ACh in crustacean nerve and nerve extract. J. Biol. Chem., *185:* 813–816.

Eccles, J. C. 1957 The Physiology of Nerve Cells. Johns Hopkins Press, Baltimore, 270 pp.

Edwards, C., and S. W. Kuffler 1957 Inhibitory mechanisms of gamma-amino-butyric acid on an isolated nerve cell. Fed. Proc., *16:* 34.

Ellis, C. H., C. H. Thienes and C. A. G. Wiersma 1942 The influence of certain drugs on the crustacean nerve-muscle system. Biol. Bull., *83:* 334–352.

Florey, E. 1954 Über die Wirkung von 5-oxytryptamin (enteramin) in der Krebschere. Z. Naturforsch., *9B:* 540–547.

——— 1957a Chemical transmission and adaptation. J. Gen. Physiol., *40:* 533–545.

——— 1957b Further evidence for the transmitter-function of Factor I. Naturwiss., *44:* 424–425.

Florey, E., and E. Florey 1953 Über die Bedeutung von 5-HT als nervöse Aktionssubstanz bei Cephalopoden und dekopoden Crustaceen. Ibid., *40:* 413–414.

——— 1954 Über die mögliche Bedeutung von Enteramin (5-oxytryptamin) als nervöse Aktionssubstanz bei Cephalopoden und Dekopoden Crustaceen. Z. Naturforsch., *9B:* 58–68.

Frontali, N. 1958 ACh synthesis in the housefly head. J. Ins. Physiol., *1:* 319–326.

Gaudin, O. 1937 Recherches sur l'action physiologique des pyrethrines. Vigot Freres, Paris.

van Harreveld, A. 1936 A physiological solution for freshwater crustaceans. Proc. Soc. Exp. Biol. Med., *34:* 428–432.

Hayashi, T. 1958 Inhibition and excitation due to gamma-aminobutyric acid in the CNS Nature, *182:* 1076–1077.

Hichar, J. K. 1960a Pulse height analysis of spontaneous activity in the crayfish central nervous system. Proc. Second Internat. Conf. on Med. Electronics. Iliffe & Sons, Ltd., London (in press).

——— 1960b Effects of gamma-aminobutyric acid and picrotoxin on spontaneous activity in the crayfish central nervous system. In preparation.

Jullien, A., and D. Vincent 1938 Sur les esters de la choline et la cholinesterase chez les crustaces. C. R. Soc. Biol., Paris, *T129:* 845–848.

van der Kloot, W. G. 1955 The control of neurosecretion and diapause by physiological changes in the brain of the *Cecropia* silkworm. Biol. Bull., *109:* 276–294.

van der Kloot, W. G., J. Robbins and I. M. Cooke 1958 Blocking by picrotoxin of peripheral inhibition of crayfish. Science, *127:* 521–522.

Maynard, D. M. 1955 Activity in a crustacean ganglion. II. Pattern and interaction in burst formation. Biol. Bull., *109:* 420–436.

Pfeiffer, C. C., E. H. Jenney, W. Gallagher, R. P. Smith, W. Bevan, Jr., K. F. Killam, E. K. Killam and W. Blackmore 1957 Stimulant effect of 2-dimethylaminoethanol—possible precursor of brain ACh. Science, *126:* 610–611.

Prosser, C. L. 1934 Action potentials in the nervous system of the crayfish. I. Spontaneous impulses. J. Cell. and Comp. Physiol., *4:* 185–209.

——— 1940 Action potentials in the nervous system of the crayfish. Effects of drugs and salts upon synaptic transmission. Ibid., *16:* 25–38.

Riker, W. F., W. C. Wescoe, McK. Cattell and E. Shorr 1946 The mechanism of action of prostigmine. Fed. Proc., *5:* 198–199.

Roeder, K. D. 1941 The effect of K and Ca on the spontaneous activity of the isolated crayfish nerve cord. J. Cell. and Comp. Physiol., *18:* 1–13.

Roeder, K. D., and S. Roeder 1939 Electrical activity in the isolated ventral nerve cord of the cockroach. I. The action of pilocarpine, nicotine, eserine and ACh. Ibid., *14:* 1–12.

Schallek, W. 1945 Action of K on bound ACh in lobster nerve cord. Ibid., *26:* 15–24.

Schallek, W., and C. A. G. Wiersma 1948 The influence of various drugs on a crustacean synapse. Ibid., *31:* 35–47.

Shore, P. A., J. A. R. Mead, R. G. Kuntzman, S. Spector and B. B. Brodie 1957 On the physiological significance of monoamine oxidase in brain. Science, *126:* 1063–1064.

Smith, R. I. 1939 ACh in the nervous tissues and blood of the crayfish. J. Cell. and Comp. Physiol., *13:* 335–344.

Spector, S., D. Prockop, P. A. Shore and B. B. Brodie 1958 Effect of iproniazid on brain levels of norepinephrine and serotonin. Science, *127:* 704.

Tobias, S. M., J. I. Kollross and I. Savit 1946 ACh and related substances in the cockroach, fly, and crayfish and the effect of DDT. J. Cell. and Comp. Physiol., *28:* 159–186.

Twarog, B. M., and K. D. Roeder 1956 Properties of the connective tissue sheath of the cockroach abdominal nerve cord. Biol. Bull., *111:* 278–286.

——— 1957 Pharmacological observations on the desheathed last abdominal ganglion of the cockroach. Ann. Ent. Soc. Amer., *50:* 231–237.

Walop, J. N. 1950 ACh formation in the central nervous system of *Carcinus maenas.* Acta Physiol. Pharmacol. Neer., *1:* 333–335.

——— 1951 Studies on ACh in the crustacean central nervous system. Arch. Int. Physiol., *59:* 145–156.

Welsh, J. H. 1954 Hydroxytryptamine: a neurohormone in the invertebrates. Fed. Proc., *13:* 162–163.

——— 1957 Serotonin as a possible neurohumoral agent: evidence obtained in lower animals. Ann. N. Y. Acad. Sci., *66:* 618–630.

Welsh, J. H., and R. Taub 1948 The action of choline and related compounds on the heart of *Venus mercenaria.* Biol. Bull., *95:* 346–353.

Woolley, D. W. 1955 Production of abnormal (psychotic?) behavior in mice with LSD, and its partial prevention with cholinergic drugs and 5-HT. Proc. Nat. Acad. Sci., *41:* 338–344.

Zeller, E. A., J. Barsky, J. R. Fouts, W. F. Kirchheimer and L. S. van Orden 1952 Influence of isonicotinic acid hydrazide (INH) and 1-isonicotinyl-2-isopropyl hydrazide (IIH) on bacterial and mammalian enzymes. Experientia, *8:* 349–350.

The Response of Tetrahymena pyriformis to Ionizing Radiation: Strain Specific Radiosensitivities

CAROLYN WELLS
Biology Division, Oak Ridge National Laboratory,[1] Oak Ridge, Tennessee

One of the fundamental problems of radiation biology is concerned with sensitivity differences among organisms. Exposure doses fatal for all members of one genus often permit a high percentage of survival of another (Bacq and Alexander, '55; Hollaender, '54). Differences in radiosensitivity at the intrageneric level are also known. To illustrate, *Pelomyxa illinoisensis* is about 10 times as radiosensitive as *Pelomyxa carolinensis* (Daniels, '55). It is further true that strains of a single species differ in this respect; a notable example is the radiation-resistant mutant, *Escherichia coli* B/r (Witkin, '46, '47).

Compared with other phyla, protozoa are relatively insensitive to the killing action of ionizing radiation (Wichterman, '57); and, among the protozoa, the ciliates are in general more radioresistant, although exceptions are known (Williams, '58). A number of reports indicate that *Tetrahymena pyriformis* is such a resistant ciliate (Ducoff, '56; Elliott and Clark, '56). Several factors warrant the use of this species in the study of radiation effects on microorganisms. First, its biochemistry and physiology has been reported in many publications (see review by Seaman, '55). Knowledge of the metabolic processes of an organism are vital for definitive understanding of many radiation effects (e.g., Stapleton, '55). Second, cytological studies of *T. pyriformis* are practical (Ray, '56); the haploid chromosome number is 5, and the chromosomes of the micronucleus may be studied in meiosis and mitosis. Thus nuclear and chromosomal consequences of ionization may be determined. Finally, since the breeding system of this species has been established (Elliott and Hayes, '53; Gruchy, '55), genetic analyses are feasible (e.g., Nanney, Caughey and Tefankjian, '55).

This study is concerned with the lethal effects of x-irradiation on 4 clonal strains of *T. pyriformis*. DNA and RNA analyses were made on mass populations of each strain; in addition, mitotic disturbances after irradiation were examined cytologically. It was established that strain differences in radiosensitivity occur in this species that cannot be correlated with either the ploidy of the micronucleus or the total DNA content of the cell. The meaning of this lack of correlation is discussed in relation to interpretations of radiosensitivity differences in this species.

METHODS

Three of the 4 clonal strains of *T. pyriformis* studied, EU 6000, EU 6001, EU 6002 (Wells, '60) are members of variety 6, mating types I, II, and III, respectively. The fourth, HS, is a micronucleate strain of unknown sexual capacity.[2] EU 6001 is presently a micronuclear tetraploid; EU 6000 and EU 6002 are diploid (Ray, '58). EU 6002 is an F_1 exconjugant progeny clone obtained by Gruchy ('55) from a cross between EU 6000 and EU 6001 when the latter was diploid. Laboratory cultures of these clones were grown without aeration at 18°C in tubes of axenic peptone-tryptone medium (Elliott and Hayes, '53). Routine 48-hour transfers of 1 ml of previous cultures were made throughout the investigation.

---

[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

[2] This strain was obtained from Dr. D. M. Prescott (see Prescott, '57) and was found to be micronucleate, contrary to other published reports (cf. Corliss '54). Subsequent investigation has shown that micronucleate cultures designated HS are not members of this strain, which is amicronucleate. A manuscript clarifying the situation has been submitted for publication elsewhere.

Stationary - phase cultures (48 ± 2 hours) were irradiated; under the conditions of growth used, less than 1% of each of these cultures was in division, and the occurrence of dead organisms resulting from food exhaustion was negligible. The stationary cultures were therefore more or less homogeneous populations of interphase organisms, and variation attributable to mixed division stages is probably small.

The x-ray source was a General Electric Maxitron 250 operated at 250 kvp and 30 ma, with 3 mm of Al filtration added to give a half-value layer of 0.43 mm of Cu. Before each run, the output of the machine was monitored with a Victoreen thimble chamber at a distance of 50 cm from target to source. The intensity at this point varied from 246 to 268 r/minute. The factor to convert these monitor readings to the intensity at the position of the organisms was obtained by chemical dosimetry with the starch-iodine method for peroxide determination (Savage, '51). The dose rate at the position of the organisms was calculated to be, on the average, 9.5 ± 0.4 kr/minute. There was no appreciable elevation of temperature during any radiation exposure.

One milliliter (ca. 10^5 cells) of a culture was irradiated in a 10-ml beaker retained by a lucite holder on top of the x-ray tube, which was inverted, directing the beam upward through the bottom of the holder and the beaker. Catalase (50 μg/ml) was added before irradiation to avoid the effects of radiation-produced peroxides. Single samples of organisms were exposed to various total doses ranging from 47.5 to 332.5 kr. Several total doses (a separate sample for each dose) were always given on the same day; irradiation was repeated on different days to obtain large total numbers of isolates at various doses.

Immediately after treatment, 50 single-cell isolations per strain per dose were made from irradiated and control (unirradiated) populations. Each cell was isolated by micropipette with the aid of a dissecting microscope into a 50-mm petri dish containing 5 ml of broth to which 1 mg/ml SRD antibiotic (a mixture of penicillin, streptomycin, and dihydrostreptomycin) had been added to inhibit contamination. For simplicity, survival was measured as the ability of the single-cell isolates to form cultures visible to the unaided eye. Isolates that did not form visible cultures were examined weekly until no living organisms could be found. If contamination occurred before termination of the experiment, the dish was discarded. At least two random samples of survivors from each experimental and control group were transferred to test tubes of broth for observation.

In one experiment, estimates of the time of cell line death (in terms of generations after irradiation) were based on observations of isolates of strains EU 6000 and EU 6002 after exposure to 0, 95, 142.5, and 190 kr. Routine examination of each isolate was made 5 days after irradiation, and the numbers of "late deaths" and "early deaths" occurring at this time were recorded. "Early deaths" were scored as those dishes containing no viable cells, and no more than 4 discernible corpses. "Late deaths" were scored as those dishes containing no viable cells and more than 4 discernible bodies. Since all cells still alive at 5 days had divided at least twice, all cell lines dying after this time were classified as "late deaths." Reexamination of late death cultures a week after classification showed that corpses disintegrate very slowly in axenic medium; certainly not enough disintegration occurs by the fifth day after irradiation to bias the results severely. Therefore, the numbers of bodies found at 5 days is probably an adequate measure of the number of animals that had been produced.

Another experiment was designed to measure fission delay after irradiation in strains EU 6000 and EU 6002. After doses of 0, 95, 190, and 285 kr, 50 single-cell isolates per dose per strain were made into 10 depression-spot plates containing broth with 1 mg/ml antibiotic added. The total number of animals in each depression was counted 24 hours after isolation. The experiment was coded to prevent subjective factors from influencing the results.

Preliminary cytological studies of radiation-induced abnormalities in mitotic micronuclei were made. Mass populations of EU 6000 and EU 6002, irradiated with 190 kr, were kept in separate petri dishes in fresh medium plus the usual concentration of antibiotic. Twenty-four hours after irradiation, these organisms were washed by centrifugation in Dryl physiological salt solution (Dryl, '59), fixed (Nissenbaum, '53), and stained in Gomori's hematoxylin (Melander and Wingstrand, '53). The cytology of the micronucleus was studied in comparison with control slides made under identical conditions. Slides of 71 cultures chosen at random from survivors of all strains at all doses were prepared as described, and examined for micronuclear abnormalities. The number of lines studied in this manner are as follows: 0 kr, 10; 47.5 kr, 9; 95 kr, 11; 142.5 kr, 5; 190 kr, 8; 237.5 kr, 11; 285 kr, 9; and 332.5 kr, 8.

Unirradiated cultures of all 4 strains were analyzed biochemically in a search for a possible basis for the observed differences in radiation sensitivity. Forty-eight-hour stationary-phase cultures, grown in 1000 ml of broth in 6000-ml Erlenmeyer flasks, were concentrated and washed in Dryl solution by centrifugation. The final concentration of organisms was estimated by fixing an aliquot in osmium tetroxide and counting the cells in an appropriate dilution with the aid of a Sedgwick-Rafter counting cell (Hall, Johnson and Loefer, '35). Acid-soluble (6% trichloroacetic acid) extracts of all strains were tested for acid-extractable -SH compounds by various methods (Dische and Zil, '51; Khym, Shapira and Doherty, '57). The total weight of the acid-insoluble fraction (primarily lipid, protein, and nucleic acid) was determined. DNA and RNA were extracted and separated by the method of Tyner, Heidelberger, and LePage ('53; see also Volkin and Cohn, '54), and measured in the Beckman spectrophotometer at 260 mμ. Duplicate cultures of each strain were analyzed biochemically.

A final experiment was designed to measure the catalase content of each of the EU strains. Washed cells were fragmented by freezing and submitted to starch gel electrophoresis. The benzidine reaction was used to test some of the electrophoretic strips for catalase; other strips were stained with amido Schwarz 10B to examine the protein patterns of each strain.[3]

RESULTS

Survival

Survival data obtained in this study are summarized in table 1 and figure 1. All data obtained at each point on the survival curves were pooled; this is justified since no statistically significant heterogeneity within dose groups was found (lowest $P = 0.10$).

Differences in radiosensitivity among the 4 strains of *T. pyriformis* are demonstrated by the survival curves of the EU strains and by the single-dose experiment of strain HS. Salient features indicated by these data are (1) EU 6000 is about twice as radiore-

---

[3] Appreciation is expressed to Dr. R. A. Popp for the electrophoretic analysis.

TABLE 1

Survival data for four clonal strains of Tetrahymena pyriformis

X-ray dose	EU 6000		EU 6001		EU 6002		HS	
	N[1]	% S[2]	N	% S	N	% S	N	% S
kr								
0	447	95.7	340	96.2	348	93.1	46	100.0
47.5	100	92.0	100	81.0	143	68.5		
95	248	89.5	199	55.3	184	53.8		
142.5	145	74.5	47	40.4	145	51.0		
190	208	70.3	49	20.4	490	27.3		
237.5	226	26.1	100	9.0	198	16.2		
285	339	1.8	48	0.0	238	0.0	139	71.2
332.5	96	0.0	49	0.0	99	0.0		

[1] Total isolates minus contaminated isolates.

[2] Percentage survival.

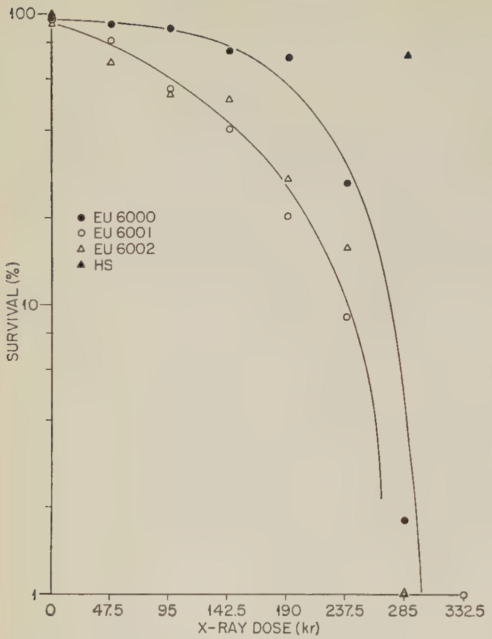


Fig. 1 Survival curves of three EU strains, and 285 kr dose experiment of strain HS.

sistant as EU 6001 and EU 6002. A comparison of strains EU 6000 and EU 6002 at each point, by the chi-square method, shows that the difference is significant (highest $P < 0.04$). (2) EU 6001 is significantly different from EU 6000 at the 95-kr point ($P = 0.001$), and *not* significantly different from EU 6002 at this point ($P < 0.77$). Thus the survival of the micronuclear tetraploid (EU 6001) is the same as that of one diploid (EU 6002), and different from that of the other (EU 6000). Limited data (see table 1) for the survival of EU 6001 at other dose points indicate a general similarity between this strain and EU 6002. (3) The survival of strain HS after 285 kr is significantly higher than that of the EU strains.

Various concentrations of SRD antibiotic used as postirradiation treatments affected the survival of these ciliates (table 2); with increasing concentration, antibiotic decreases the yield of radiation survivors, but there is no evidence that this is a differential effect among strains. The concentration of 1 mg/ml used in this work did not decrease survival enough to be of any significance for the interpretation of the results.

Intermediate events

The preceding section gives the results only in terms of final survival. The events between irradiation and either death or survival are also noteworthy. Some of the major questions are: When does death occur in relation to fissions after irradiation? At what time after irradiation do radiation-induced cell deaths cease? Does fission delay occur after x-irradiation? What nuclear damage results from radiation?

Time of death. Two types of death are characteristic of *T. pyriformis* after irradiation. Frequencies of these two types for two strains are shown in table 3.

One type of radiation-induced death (early death) is responsible for a large portion of the total death of EU 6002, but only for a small amount of that of EU 6000. Data from single-cell isolations to be presented in the next section suggest that most early deaths occurred after at least one cell division.

TABLE 2

The effect of various concentrations of SRD antibiotic used as posttreatments on the survival of strains EU 6000 and EU 6002 after 0 and 190 kr

SRD	EU 6000				EU 6002			
	0 kr		190 kr		0 kr		190 kr	
	N[1]	% S[2]	N	% S	N	% S	N	% S
mg/ml								
0	41	98	42	71	45	100	40	30
1	50	96	49	69	50	92	50	26
2	50	92	50	60	50	94	50	22
3	50	98	50	28	50	88	50	14

[1] Total isolates minus contaminated isolates.
[2] Percentage survival in this particular experiment.

TABLE 3

Early death and late death of two clonal strains of T. pyriformis after various x-ray doses

X-ray dose	EU 6000					EU 6002				
	Early death	Late death$_5$[1]	Late death$_{>5}$[2]	Survival %[3]	Total isolates[4]	Early death	Late death$_5$	Late death$_{>5}$	Survival %	Total isolates
kr										
0	2	0	0	98	100	2	0	0	98	100
95	0	2	12	88	98	28	0	8	62	96
142.5	2	1	23	74	100	31	2	20	46	98
190	6	3	26	65	100	42	4	28	26	100

[1] Observed 5 days after irradiation.
[2] Occurring later than 5 days after irradiation.
[3] In this particular experiment.
[4] Minus contaminated isolates.

Late death accounts for almost all of the death of strain EU 6000 but for only a portion of that of strain EU 6002. It is notable that most late death cultures, of both strains and after all doses, contained many more than 4 bodies, in fact usually enough to account for at least 8 generations (often more than 256 cells). Only a minor portion of the ultimate total late death had occurred at 5 days. Since many cell lines were capable of numerous divisions before death, *T. pyriformis* is different from some other ciliates (Kimball, '57) that do not divide before death.

No systematic survey of this type was made on strain EU 6001 or on strain HS. However, a few observations of dead cultures of HS, after 285 kr, indicate that at least 8 fissions had occurred before some cell lines died.

The time required for the formation of visible cultures from single-cell isolates may also be mentioned. Control isolates established such cultures in 5 days (20–30 fissions) after isolation. Irradiated isolates were variable; some cultures were macroscopically visible as soon as controls, a few required as long as 20 days to become so. This variation was found among isolates of a single dose as well as among those of different doses. Nevertheless, the higher-dose groups took longer, on the average, to become established. The trend was toward a correlation between the final percentage survival and the time for establishment of visible clones, not between this time and total dose. A striking example is seen by comparing strains HS and EU 6000 after 285 kr. Practically all the survivors (71%) of HS were countable *before* the few survivors (1.8%) of EU 6000.

Once the single-cell isolates had divided enough times to become visible to the unaided eye, the line of descent did not die. Random samples of surviving cultures of each strain, and at each dose, have grown well in test tube culture for many months. With one exception, these cultures and the unirradiated controls have not differed in density of population produced per unit of inoculum. One clone of EU 6000 surviving 285 kr has failed to grow as well as controls for several months, but there is no indication that the line will be lost.

Fission delay. A third measure of events between irradiation and termination of the experiment is the fission delay during the first 24 hours (table 4). Division delay was significant after doses of 190 and 285 kr in both EU 6000 and EU 6002. The data for EU 6000 suggest a threshold for fission delay of at least 95 kr, followed by a gradual increase in rate of generation time with progressively higher doses. In EU 6002, the drop was more precipitous, to about 1.5 fissions after 190 kr, with no further delay with dose. Thus, by this as well as other criteria, EU 6002 is more sensitive to radiation.

The fission delay experiments also showed that little death occurs during the first 24 hours, even after doses that ultimately cause a high percentage of death. It is clear that most lines that eventually die undergo at least one division, often more; this is substantiated by the obser-

TABLE 4

Frequency distribution and average number of generations of 50 isolates per dose of two clonal strains of T. pyriformis 24 hours after irradiation with various doses

X-ray dose	D[1]	No. of generations at 24 hours						Average generations per day
		0	1	2	3	4	5	
kr								
				EU 6000				
0	1	5	1	1	3	31	8	3.59
95	2	3	0	1	16	26	2	3.42
190	5	4	3	8	23	6	1	2.60
285	1	8	6	27	5	3	0	1.78
				EU 6002				
0	0	1	1	9	13	23	3	3.30
95	1	6	0	0	6	30	7	3.53
190	3	14	15	10	8	0	0	1.25
285	2	5	13	27	3	0	0	1.58

[1] Number of depressions in which no animals were found.

vations in petri dish cultures 5 days after irradiation.

Nuclear damage. Although cytological studies of radiation-induced nuclear abnormalities are preliminary, several points may be made. Each of the 71 clones that survived various doses was micronucleate. This representative sample of all the survivors scored throughout the investigation included members of every strain at each dose. In many cases, the micronucleus of survivors appeared less compact than that of controls, but the functional ability of the irradiated micronuclei seemed to be unimpaired, i.e., mitotic figures during fission were no different from those of controls, no aberrations were observed, and spindles were not disoriented. No macronuclear damage was observable.

On the other hand, examination of mass cultures of EU 6000 and EU 6002 24 hours after 190 kr of radiation demonstrated the presence of micronuclear abnormalities in many members of the population. In general, micronuclei of EU 6002 were more severely damaged than those of EU 6000, a correlation with their different radiosensitivities. Many micronuclei in both strains were disoriented to such an extent that one sister product of fission was rendered amicronucleate (fig. 2). Amicronucleate organisms were commonly found on slide preparations of experimental organisms, more frequently in EU 6002. No obvious morphological damage to the macronucleus was observed.

Biochemical studies of control populations

Finally, certain biochemical analyses of these 4 strains have been made; the results are tabulated in table 5. The weights of the acid-insoluble fractions of each strain indicate that strain HS is smaller than the EU strains. This is supported by visual observations made by D. M. Prescott on the cell sizes of these same strains (personal communication).

Nucleic acids. Strain HS, the most resistant to radiation of all 4 strains examined, has significantly less DNA per cell than the EU strains. The RNA per cell of all strains is equivalent; the slight discrepancy among strains with respect to total units of RNA per 10^7 cells (or per weight) is within the limits of error of the method used (E. Volkin, personal communication). The predominance of RNA in the total nucleic acid fraction is consistent with findings in other strains of *T. pyriformis* (Heinrich et al., '52; Flavin and Engelman, '53; Scherbaum, '57).

Acid-soluble sulfhydryl. No free sulfhydryl was found in the acid-soluble fraction of any of the strains, although, under the conditions of the experiment, levels as low as 6.7×10^{-14} mole of S/cell could have been detected. The meaning of this finding is obscure at present.

Electrophoretic patterns. The catalase content of each of the EU strains is equiv-

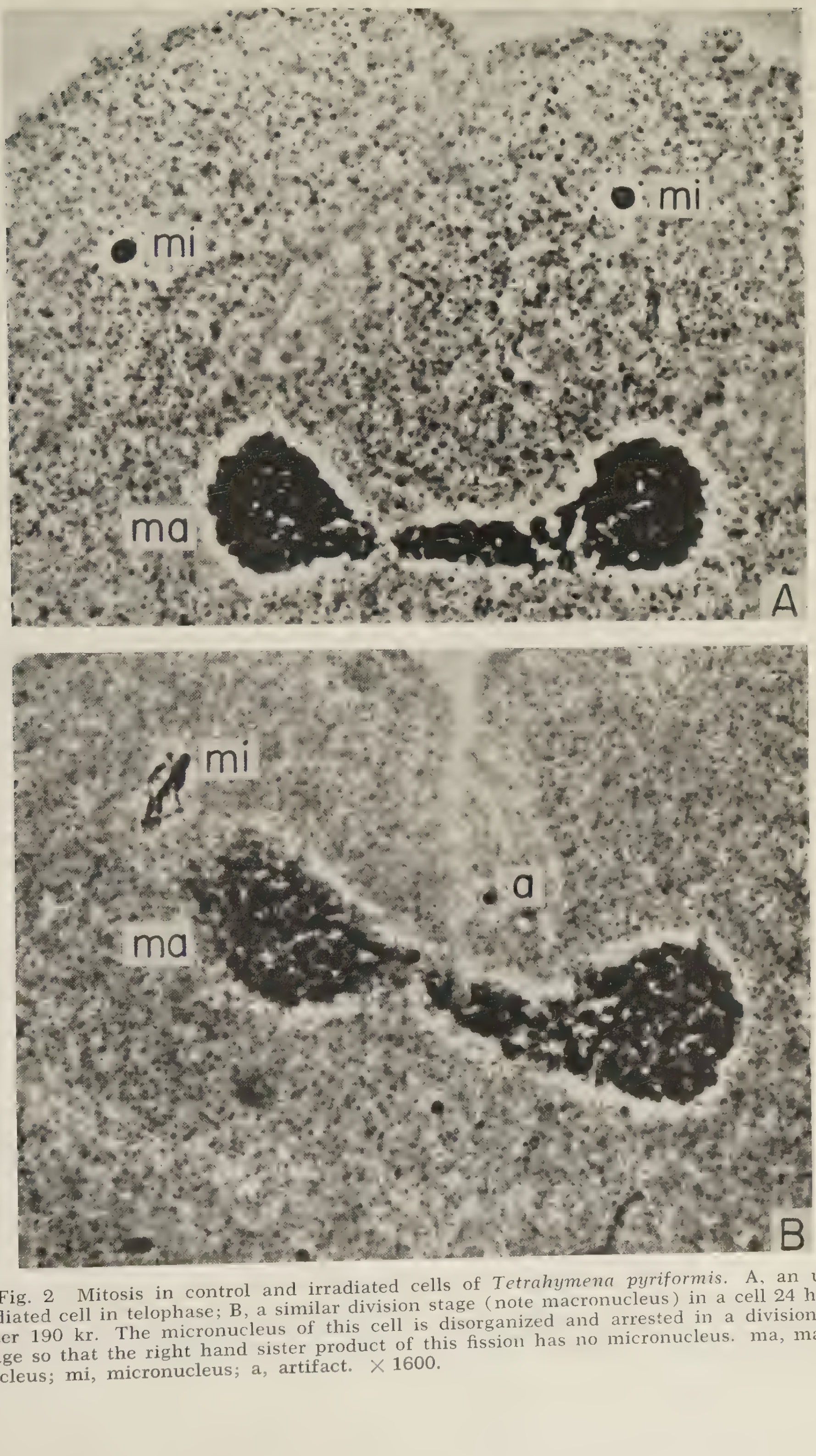


Fig. 2 Mitosis in control and irradiated cells of *Tetrahymena pyriformis*. A, an unirradiated cell in telophase; B, a similar division stage (note macronucleus) in a cell 24 hours after 190 kr. The micronucleus of this cell is disorganized and arrested in a division-like stage so that the right hand sister product of this fission has no micronucleus. ma, macronucleus; mi, micronucleus; a, artifact. × 1600.

TABLE 5

Biochemical data obtained for all 4 clonal strains studied in this investigation (Units of nucleic acid calculated from Beckman spectrophotometer readings at 260 mμ)

Strain	DNA		RNA		Acid-insol. fraction, g/10^7 cells
	Units/10^7 cells	Units/g of a.i.f.[1]	Units/10^7 cells	Units/g of a.i.f.	
EU 6000	4.1 3.8	14.5 13.8	104.7 103.5	362.0 369.6	0.289 0.280
EU 6001	4.5 4.1	15.1 14.4	96.8 99.2	336.1 348.8	0.288 0.284
EU 6002	3.9 3.9	14.4 14.0	97.0 97.8	354.4 349.2	0.274 0.280
HS	2.0 2.1	8.4 8.2	99.8 100.9	390.0 387.7	0.238 0.256

[1] Acid-insoluble fraction.

alent, i.e., no differences in intensity of the stained catalase band were apparent among strains. When the electrophoretic strips were stained with the protein dye, amido Schwarz 10B, 13 bands per strain were observed. Only one difference among strains was indicated. This was a quantitative difference, shown by the various staining intensities of a single negatively charged protein band. EU 6002 seemed to contain the greatest amount of this protein, EU 6001 the least amount, and EU 6000 an intermediate amount. There is no apparent correlation between the amount of this unknown protein and the radiosensitivities of the EU strains.

DISCUSSION

The differences in sensitivity among the clonal strains of *T. pyriformis* allow some distinction among various possible mechanisms of radiation killing. Briefly, differences in sensitivity can arise from differences in target multiplicity, target size, amount of protection against initial injury, or amount of restoration of this injury (see discussions in Gray, '54; Laterjet, '54; Laterjet and Gray, '54; Hollaender, '54; Lea, '55). The combined biochemical, cytological, and survival data reported in this paper, though not leading to a final choice of mechanism, do allow the exclusion of a number of hypotheses that otherwise might have appeared probable.

The death of a cell after irradiation may depend on the inactivation of more than one target within the cell; therefore, radiosensitivity differences among cell populations may be attributable to differences in target multiplicity. These sensitive units may be located in the cytoplasm or in the nucleus of the cell, and, in the case of the ciliates, in the micro- or macronucleus. Since much of the present data have direct bearing on the postulated existence of targets of different multiplicities in different strains, a careful consideration of the results in relation to each of the possible locations of sensitive multiple targets is desirable.

The micronucleus

In the first place, it is conceivable that radiosensitivity differences may be correlated with multiplicity differences in the micronucleus. If this multiplicity is synonymous with the ploidy of the micronucleus, however, the results do not support this interpretation. Among the EU strains, two are micronuclear diploids and one is a micronuclear tetraploid. On the above hypothesis, the tetraploid should be more radioresistant than the diploids (see, however, Mortimer, '58). This is not the case. One diploid, EU 6000, is significantly more radioresistant than the tetraploid, EU 6001; furthermore, the other diploid, EU 6002, is less resistant than EU 6000, and is no different in radiosensitivity from the tetraploid. These points are in no way compatible with the hypothesis that radiosensitivity and genome multiplicity are correlated. Evidence is also available showing that resistance to ionizing radiation does not increase with micronuclear number. The ciliate *Spathidium spathula* is multimicronucleate, yet it is extremely radiosensitive (Williams, '58). We can only conclude that something unrelated to either the multiplicity of the micronuclear genome or the number of micronuclei in the cell is responsible for differences in radiosensitivity.

Although micronuclear multiplicity, within the terms of reference just discussed, cannot be the primary cause of different radiosensitivities, the cytological studies of irradiated nuclei show a correlation between micronuclear damage and postirradiation lethality, viz., strain EU 6002, more radiosensitive than EU 6000, also suffered greater amounts of micronuclear disorganization after 190 kr than did the latter strain. This difference is not related to the micronuclear ploidy of these strains, since they are both diploid. The micronuclear disorganization experienced in greater or lesser quantity by these strains had the following characteristics. In general, the damaged micronuclei lost the typical compactness of the unirradiated micronucleus (fig. 2A), the spindle was often disoriented, and the nucleus failed to divide in many cells (fig. 2B). Only one anaphase bridge and little evidence of fragmentation was observed. As a result of the disorientation of the spindle, and of the failure of the nucleus to divide, many sister products of fission were amicronucleate; this condition was more prevalent in EU 6002 than in EU 6000.

Can this difference in amount of micronuclear damage account for the difference in viability or is it only another symptom of some more basic damage? The observations on surviving clones of each strain and after every dose show that none of the survivors are amicronucleate. Thus the production of amicronucleates is regularly associated with death, if not itself the cause of death. A similar finding has been reported by Nanney ('59) for spontaneous inviability in *T. pyriformis*. On the other hand, viable amicronucleate clones of this species have been isolated from nature many times (Corliss, '54), and viable amicronucleates have been obtained for both *T. pyriformis* (Speidel and Holz, '59) and *Paramecium multimicronucleatum* (Wichterman, '59) after massive irradiation. It therefore seems possible that both spontaneous and radiation-induced loss of micronuclei are secondary consequences of some damage that is also lethal to the line. Precedent for this interpretation is found in reports of micronuclear abnormalities associated with aging in *P. aurelia* (Sonneborn and Schneller, '55; Dippell, '55).

It is important to bear in mind that, although the micronuclei of some irradiated cells were definitely damaged, many cells contained little or no micronuclear injury. These undamaged nuclei were found not only on slides of mass populations taken 24 hours after irradiation but also on preparations of surviving lines. In fact, each of the 71 survivors examined was characterized by cells containing nuclei no different from those of unirradiated controls, except for an occasional slight loss of typical micronuclear compactness. It is remarkable that so little damage is done to ciliate micronuclei by such high doses of radiation. There is also genetic evidence that major chromosome aberrations are infrequent or lacking at lower doses, which produce many recessive lethals in *P. aurelia* (Kimball, Gaither and Wilson, '57). All of the evidence strongly suggests that the ciliate micronucleus is very efficiently protected against damage leading to chromosome aberrations, because restitutional reunion is extremely frequent or for other reasons. The foregoing findings support the conclusion that the micronuclear damage observed is not primary, i.e., it is not caused by the production of classical chromosome aberrations nor is it correlated with the ploidy of the micronucleus.

The macronucleus

It is conceivable that the large macronucleus of ciliates is the main target of radiation (see discussions by Giese, '53; Brandt and Giese, '56; Powers and Ehret, '56; Kimball, '57). The great quantity of DNA in the macronucleus (relative to the DNA of the micronucleus), whether or not organized as a large number of sensitive subunits (see Allen and Nanney, '58) could necessitate the production of many hits within it to bring about inactivation of the cell. Organisms with such a structure would be very radioresistant. Ciliates are in general resistant, as expected from this point of view, but evidence from the present and other studies show that, like micronuclear multiplicity, macronuclear multiplicity and macronuclear size are improbable bases for interpretation of variation in radiosensitivity among the ciliates.

In the first place, the total DNA determinations of the various strains of *T. pyriformis* have shown that strain HS, more resistant than the EU strains, has only half as much total DNA. Second, the differences in radiosensitivity within the EU group are not correlated with differences in total DNA content of these strains, since, in this case, no DNA differences at all were found. In relation to the target size theory, it may be further pointed out that the very sensitive species, *S. spathula,* (Williams, '58) has a macronucleus of about the same size relative to total cell volume as the macronucleus of more resistant species.

It is tempting to try to analyze radiation-induced death on the basis of the theory of macronuclear subunits (Allen and Nanney, '58; Schensted, '58). Delayed death could result from the segregation, through fission, of aberrant subunits that, while all contained within the same macronucleus, could complement each other in some way and allow temporary survival of cells (Kimball and Householder, '54; Bridgman and Kimball, '54; Nanney, '59). Segregation of replicated units would, in some cases, result in homozygosity of lethal units and delayed death. In other cases, however, the stabilization of macronuclei should result in the formation of viable cell lines, homozygous for undamaged subunits. The result of this would be a relatively long delay before final death, and, unless some threshold phenomenon were involved, the hypothesis would also predict a more or less continuous distribution in time of the appearance of visible (thus viable) cultures. Although most survivors fall into such a continuous distribution, an appreciable minority are much more delayed than the rest, suggesting that the hypothesis is not acceptable without modification.

The results obtained for strain HS are also not in agreement with the hypothesis of multiple macronuclear units. It is logical to assume that strain HS has fewer subunits than the EU strains, since it has less DNA. Thus HS should not only be more sensitive to radiation, having fewer multiple targets to inactivate, but it should also experience late death earlier, in number of generations, than the EU strains. Preliminary observations show that this is not the case; enough bodies to account for at least 8 generations have been recorded in a few 5-day-old isolates of strain HS after 285 kr.

Finally, the segregation of multiple subunits does not allow for the early death that is characteristic of strain EU 6002, even at relatively low doses. It may also be pointed out that the hypothesis is not applicable to other ciliates in relation to radiation death. Delayed death, a necessary corollary to macronuclear multiplicity, is not found in *P. aurelia* after x-irradiation (Kimball, Geckler and Gaither, '52). In similar studies with an amicronucleate strain of *Tillina magna,* Bridgman and Kimball ('54) report that cells that die have depressed fission rates in the first few days after irradiation; those that survive are depressed less. This connection between early effects and late death is difficult or impossible to explain on a macronuclear segregation hypothesis, as the authors observe.

The cytoplasm

Assuming, then, that the multiplicity, or size, of the nuclei cannot account for the observed lethal effects of ionizing radiation, we are left with the possibilities that the multiplicity, or size, of some cytoplasmic target is the mechanism site, or that protection or repair mechanisms are operative. Although a cytoplasmic basis for certain vegetative effects of x-rays has been suggested by Powers ('55), and Powers and Ehret ('56) for *Paramecium,* little evidence from the present studies has direct bearing. It may be indicated in passing that members of strain HS seem to be smaller than those of the EU strains, thus these organisms should be more sensitive than larger cells, on a cytoplasmic multiple target basis. The observation that HS is more resistant does not exclude other possible cytoplasmic interpretations of killing, however.

Other interpretations

Stimulated by the findings that -SH compounds act as protective agents against radiation damage in certain systems (see Hollaender, '56), efforts were made to measure the free -SH content of the various strains under investigation. The failure to detect such compounds in acid-soluble extracts cannot exclude the possibility that protective agents may be operating in this system, affording greater protection to the more resistant strains. The protective capacity of catalase in destroying radiation-produced peroxides (Adler, '58) does not account for the differences among EU strains, since electrophoresis revealed no differences in catalase content among them and since supplementary catalase was added to all samples before irradiation.

In addition, the protein patterns obtained after electrophoresis showed no differences among the EU strains that can be correlated with radiosensitivity. One protein band was quantitatively different for each EU strain, but the radioresistant strain, EU 6000, was intermediate between the radiosensitive strains EU 6001 and EU 6002 in this respect. Therefore, this protein difference cannot represent any protective substance.

Repair mechanisms also afford possible explanations for the observed differences in radiosensitivity. The therapeutic action of unirradiated cytoplasmic fractions on irradiated *Pelomyxa* (Daniels, '58) suggests that repair of the damaged cell may be brought about by particles within the cytoplasm itself, if these particles are not irrevocably inactivated. Although the hypothesis of a repair mechanism as an interpretation of radiation lethality in the ciliates must await experimental substantiation before it can be discussed meaningfully, such an interpretation is a possible way by which the variable results obtained among ciliate species could be unified.

In closing, it should be pointed out that many of the results of the present investigation closely resemble the "cellular accidents" that Nanney ('59) described in *T. pyriformis*. He observed the spontaneous origin of nonviable and otherwise aberrant cell lines. These cell lines occurred with different frequencies in different clones. Radiation might be assumed to increase the frequency of such "accidents," but since the nature of neither the spontaneous nor the radiation-induced "accidents" is known the idea is not very helpful. The present work, together with other studies, serves, however, to delimit the possibilities, and thus brings us closer to a true understanding of this form of radiation damage.

SUMMARY

1. Four clonal strains, HS, EU 6000, EU 6001, and EU 6002, of *T. pyriformis* were exposed to x-ray doses ranging from 48 to 333 kr. After irradiation, single cell isolations were made. Survival was measured as the ability of these single cell isolates to form macroscopically visible colonies. By this technique, it was found that some of these strains are more radioresistant than others.

2. Certain events between treatment and termination of the experiment (either by death or survival of the cell lines) were studied. Death may occur in some cases during the first two generations, and in other cases after the cells have divided many times. The proportions of these two types of death are different for strains of different radiosensitivities. Fission delay during the first 24 hours after irradiation increased with increasing dose. Strains of different radiosensitivities, as measured by survival, were also different with respect to radiation-induced fission delay.

3. Cytological studies of irradiated cells show certain micronuclear abnormalities not observed in controls. The production of these abnormalities, more frequent in EU 6002 than in EU 6000, often leads to amicronucleate sister products of fission.

4. All 4 strains were analyzed biochemically. The most radioresistant strain, HS, had only half as much total DNA per cell as the more radiosensitive EU strains. The RNA content was equivalent.

5. Certain interpretations of radiation sensitivity differences are excluded by the data. The radiosensitivity of the strain is not correlated with the ploidy of the micronucleus. Also, no correlation can be made with the total DNA content of the strains.

These and other points are discussed showing that interpretations based on differences in target multiplicity are not compatible with the results. It is suggested that radiosensitivity differences are related to differences in ability of the strains either to protect against radiation damage or to repair damage that has been done. A choice between these interpretations is not allowed by the present data.

LITERATURE CITED

Adler, H. I. 1958 An aftereffect of ionizing radiation on a mutant of *Escherichia coli*. Radiation Research, *9:* 451–458.

Allen, S. L., and D. L. Nanney 1958 An analysis of nuclear differentiation in the selfers of *Tetrahymena*. Am. Naturalist, *92:* 139–160.

Bacq, Z. M., and P. Alexander 1955 Fundamentals of Radiobiology. Academic Press, Inc., New York.

Brandt, C. L., and A. C. Giese 1956 Photoreversal of nuclear and cytoplasmic effects of short ultraviolet radiation on *Paramecium caudatum*. J. Gen. Physiol., *39:* 735–751.

Bridgman, J., and R. F. Kimball 1954 The effects of X rays on division rate and survival of *Tillina magna* and *Colpoda* sp. with an account of delayed death. J. Cell. and Comp. Physiol., *44:* 431–445.

Corliss, J. O. 1954 The literature on *Tetrahymena*: Its history, growth, and recent trends. J. Protozool., *1:* 156–169.

Daniels, E. W. 1955 X-irradiation of the giant amoeba, *Pelomyxa illinoisensis*. I. Survival and cell division following exposure. Therapeutic effects of whole protoplasm. J. Exp. Zool., *130:* 183–197.

——— 1958 X-irradiation of the giant amoeba, *Pelomyxa illinoisensis*. II. Further studies on recovery following supralethal exposure. Ibid., *137:* 425–442.

Dippell, R. V. 1955 Some cytological aspects of aging in variety 4 of *Paramecium aurelia*. J. Protozool., 2, Suppl.: 7.

Dische, Z., and H. Zil 1951 Studies on the oxidation of cysteine to cystine in lens proteins during cataract formation. Am. J. Opthalmol., *34* (Pt. II): 104–113.

Dryl, S. 1959 Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. J. Protozool., 6, Suppl.: 25.

Ducoff, H. S. 1956 Radiation-induced fission block in synchronized cultures of *Tetrahymena pyriformis* W. Exp. Cell Res., *11:* 218–220.

Elliott, A. M., and G. M. Clark 1956 The induction of haploidy in *Tetrahymena pyriformis* following X-irradiation. J. Protozool., *3:* 181–188.

Elliott, A. M., and R. E. Hayes 1953 Mating types in *Tetrahymena*. Biol. Bull., *105:* 269–284.

Flavin, M., and M. Engelman 1953 Aminopurine interconversion in *Tetrahymena geleii*: role of 8-azaguanine and hypoxanthine. J. Biol. Chem., *200:* 59–68.

Giese, A. C. 1953 Protozoa in photobiological research. Physiol. Zool., *26:* 1–22.

Gray, L. H. 1954 Conditions which affect the biologic damage resulting from exposure to ionizing radiation. Acta Radiol., *41:* 63–83.

Gruchy, D. F. 1955 The breeding system and distribution of *Tetrahymena pyriformis*. J. Protozool., *2:* 178–185.

Hall, R. P., D. F. Johnson and J. B. Loefer 1935 A method for counting protozoa in the measurement of growth under experimental conditions. Trans. Am. Microscop. Soc., *54:* 298–300.

Heinrich, M. R., V. C. Dewey, R. E. Parks, Jr. and G. W. Kidder 1952 The incorporation of 8-azaguanine into the nucleic acid of *Tetrahymena geleii*. J. Biol. Chem., *197:* 199–204.

Hollaender, A., (Ed.) 1954 Radiation Biology, vol. I. McGraw-Hill Book Co., Inc., New York.

——— 1956 Modification of radiation response. In: Peaceful Uses of Atomic Energy, Geneva, August 1955, vol. 16. United Nations, New York, pp. 106–113.

Khym, J. X., R. Shapira and D. G. Doherty 1957 Ion-exchange studies of transguanylation reactions. I. Rearrangement of S,2-aminoethylisothiourea to 2-mercaptoethylguanidine and 2-aminothiazoline. J. Am. Chem. Soc., *79:* 5663–5666.

Kimball, A. W., and A. S. Householder 1954 A stochastic model for the selection of macronuclear units in *Paramecium* growth. Biometrics, *10:* 361–374.

Kimball, R. F. 1957 Nongenetic effects of radiation on microorganisms. Ann. Rev. Microbiol., *11:* 199–220.

Kimball, R. F., N. Gaither and S. M. Wilson 1957 Postirradiation modification of mutagenesis in *Paramecium* by streptomycin. Genetics, *42:* 661–669.

Kimball, R. F., R. P. Geckler and N. Gaither 1952 Division delay by radiation and nitrogen mustard in *Paramecium*. J. Cell. and Comp. Physiol., *40:* 427–459.

Laterjet, R. 1954 Spontaneous and induced cell restorations after treatments with ionizing and non-ionizing radiations. Acta Radiol., *41:* 84–100.

Laterjet, R., and L. H. Gray 1954 Definition of the terms “protection” and “restoration.” Acta Radiol., *41:* 61–62.

Lea, D. E. 1955 Actions of Radiations on Living Cells, 2nd ed. Cambridge at the University Press.

Melander, Y., and K. G. Wingstrand 1953 Gomori’s haematoxylin as a chromosome stain. Stain Technol., *28:* 217–223.

Mortimer, R. K. 1958 Radiobiological and genetic studies on a polyploid series (haploid to hexaploid) of *Saccharomyces cerevisiae*. Radiation Research, *9:* 312–326.

Nanney, D. L. 1959 Vegetative mutants and clonal senility in *Tetrahymena*. J. Protozool., *6:* 171–177.

Nanney, D. L., P. A. Caughey and A. Tefankjian 1955 The genetic control of mating type potentialities in *Tetrahymena pyriformis*. Genetics, *40:* 668–680.

Nissenbaum, G. 1953 A combined method for the rapid fixation and adhesion of ciliates and flagellates. Science, *118:* 31–32.

Powers, E. L. 1955 Radiation effects in *Paramecium*. Ann. N. Y. Acad. Sci., *59:* 619–637.

Powers, E. L., and C. F. Ehret 1956 Radiation effects on cells: genetics or physiology? In: Peaceful Uses of Atomic Energy, Proc. Intern. Conf., Geneva, August 1955, vol. II. United Nations, New York, pp. 266–271.

Prescott, D. M. 1957 Relation between multiplication rate and temperature in *Tetrahymena pyriformis*, strains HS and GL. J. Protozool., *4:* 252–256.

Ray, C., Jr. 1956 Meiosis and nuclear behavior in *Tetrahymena pyriformis*. Ibid., *3:* 88–96.

——— 1958 Tetraploidy in *Tetrahymena pyriformis*. Proc. X Intern. Congr. Genetics, vol. 2. University of Toronto Press, Toronto, pp. 229–230.

Savage, D. J. 1951 The determination of hydrogen peroxide in radiation experiments on aqueous solutions. Analyst, *76:* 224–226.

Schensted, I. V. 1958 Model of subnuclear segregation in the macronucleus of ciliates. Am. Naturalist, *92:* 161–170.

Scherbaum, O. 1957 The content and composition of nucleic acids in normal and synchronously dividing mass cultures of *Tetrahymena pyriformis*. Exp. Cell Res., *13:* 24–30.

Seaman, G. R. 1955 Metabolism of free-living ciliates. In: Biochemistry and Physiology of Protozoa, vol. II. S. H. Hutner and A. Lwoff, eds. Academic Press, Inc., New York, pp. 91–158.

Sonneborn, T. M., and M. Schneller 1955 The basis of aging in variety 4 of *Paramecium aurelia*. J. Protozool., 2, Suppl.: 6.

Speidel, C. C., and G. G. Holz, Jr. 1959 Motion pictures of mating behavior of *Tetrahymena pyriformis* rendered amicronucleate by X-ray treatments. Biol. Bull., *117:* 384–385.

Stapleton, G. E. 1955 The influence of pretreatments and posttreatments on bacterial inactivation by ionizing radiation. Ann. N. Y. Acad. Sci., *59:* 604–618.

Tyner, E. P., C. Heidelberger and G. A. LePage 1953 Intracellular distribution of radioactivity in nucleic acid nucleotides and proteins following simultaneous administration of P^{32} and glycine-2-C^{14}. Cancer Res., *13:* 186–203.

Volkin, E., and W. E. Cohn 1954 Estimation of nucleic acids. In: Methods of Biochemical Analysis, vol. I. D. Glick, ed. Interscience Publishers, New York, pp. 287–305.

Wells, C. 1960 Identification of free and bound amino acids in three strains of *Tetrahymena pyriformis* using paper chromatography. J. Protozool., *7:* 7–10.

Wichterman, R. 1957 Biological effects of radiations on protozoa. Bios, *28:* 3–20.

——— 1959 Mutation in the protozoan *Paramecium multimicronucleatum* as a result of X-irradiation. Science, *129:* 207–208.

Williams, D. B. 1958 Effects of X rays on fission in the predaceous holotrich, *Spathidium spathula*. J. Protozool., 5, Suppl.: 25.

Witkin, E. M. 1946 Inherited differences in sensitivity to radiation in *Escherichia coli*, Proc. Natl. Acad. Sci. U. S., *32:* 59–68.

——— 1947 Genetics of resistance to radiation in *Escherichia coli*. Genetics, *32:* 221–248.

SYMPOSIUM ON ENZYME REACTION MECHANISMS

Sponsored by

THE BIOLOGY DIVISION
OAK RIDGE NATIONAL LABORATORY

The papers originating from this Symposium are made available to scientific circles for the first time as a Supplement issue to the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY. The publication is identified as *Supplement 1, volume 54, Journal of Cellular and Comparative Physiology,* is available without added cost to subscribers for 1959 volumes 53–54 and if requested with new subscription orders for the 1960 volumes 55–56. Single copies of Supplement issues are not for sale but are available only to subscribers of the journal. *Information concerning reprints may be obtained by addressing The Biology Division, Oak Ridge National Laboratory.*

CONTENTS

Introduction. By Alexander Hollaender.

Introduction to the symposium on enzyme reaction mechanisms. By Alexander R. Todd.

Synthesis and structural analysis of polynucleotides. Seven figures. By H. Gobind Khorana.

Mechanisms of enzymic cleavage of some organic phosphates. Five figures. By Mildred Cohn.

Participation of acyl—CoA in carbon chain biosynthesis. Twenty-five figures. By Feodor Lynen.

Carboxylations and decarboxylations. Thirty figures. By Melvin Calvin and Ning G. Pon.

Amino acid activation and protein synthesis. Seven figures. By Fritz Lipmann, W. C. Hülsmann, G. Hartmann, Hans G. Boman and George Acs.

Aldol and ketol condensations. Twenty-two figures. By Bernard L. Horecker.

Mechanisms of formylation and hydroxymethylation reactions. Eighteen figures. By F. M. Huennekens, H. R. Whitely, and M. J. Osborn.

Substrate specificity of chain propagation steps in saccharide synthesis. Two figures. By Shlomo Hestrin.

Reactions involving the carbon — nitrogen bond: heterocyclic compounds. Sixteen figures. By John M. Buchanan, Standish C. Hartmann, Robert L. Herrmann, and Richard A. Day.

The mechanism of the transamination reaction. Twelve figures. By Esmond E. Snell and W. Terry Jenkins.

The hydrolysis of peptide and ester bonds by proteolytic enzymes. Eleven figures. By Hans Neurath and Brian S. Hartley.

The chemical structure of chymotrypsin. One figure. By Brian S. Hartley.

Comments on the modification of enzymes, with special reference to ribonuclease. By Frederic M. Richards.

Some approaches to the study of active centers. Three figures. By Christian B. Anfinsen.

The aminoacyl insertion reaction. Nine figures. By Max Brenner.

The active site of esterases. Eight figures. By J. A. Cohen, R. A. Oosterbaan, H. S. Jansz, and F. Berends.

Enzyme flexibility and enzyme action. Nine figures. By Daniel E. Koshland, Jr.

Summarizing remarks. By Philip Handler.

Edition limited, no single copy sales — Subscribe now

THE PRESS OF THE WISTAR INSTITUTE
3631 SPRUCE STREET
PHILADELPHIA 4, PA.

Please send copy of Supplement 1 to volume 54 and enter my subscription to JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, 1959 volumes 53–54 and/or 1960 volumes 55–56 to include other supplements as published in 1960.

NAME

STREET

CITY ZONE STATE

Annual subscription price in U.S., $20.00; all other countries, $21.00

Original first edition back issues of

Journal of Cellular and Comparative PHYSIOLOGY

Complete volumes	*Year*	*Each volume*
1	1932	$15.00
2	1932–33	15.00
4	1933–34	10.00
5	1934–35	10.00
6	1935	10.00
7	1935–36	10.00
8	1936	10.00
30	1947	5.00
34	1948	7.50
40	1952	7.50
41	1953	7.50
42	1953	7.50
44	1954	7.50
46	1955	7.50
48	1956	7.50
50	1957	7.50
51	1958	10.00
52	1958	10.00

Incomplete volumes	*Year*	*Each number*
3, Nos. 1, 3, 4	1933	$5.00
11, No. 1	1938	2.75
12, Nos. 2, 3	1938	3.50
14, Nos. 2, 3	1939	2.75
18, No. 3	1941	2.00
28, No. 3	1946	2.00
29, Nos. 2, 3	1947	2.00
36, No. 1	1950	3.00
39, No. 1	1952	3.00
43, No. 1	1954	3.00
45, No. 3	1955	3.00
47, No. 1	1956	3.00
49, No. 3	1957	3.00

Note: Unlisted volumes and single numbers are now out of print.

1959 volumes 53–54
6 issues, $20.00

Prices subject to change without notice. Availability depends upon prior sales (issued October 15, 1959)

THE PRESS OF THE WISTAR INSTITUTE
3631 SPRUCE STREET
PHILADELPHIA 4, PA.

You may send the following issues from above listing. Complete volumes ____________________

Incomplete volumes ____________________

NAME ____________________

STREET ____________________

CITY __________ ZONE __________ STATE __________

NOTICE TO CONTRIBUTORS

The Journal of Cellular and Comparative Physiology, appearing bimonthly, is intended as a medium for the publication of papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects. Short preliminary notices are not desired and papers will not be accepted for simultaneous publication or which have been previously published elsewhere. While not specifically excluding any particular branch of physiology, contributors should recognize that excellent journals already exist for publication in the field of experimental and physiological zoology, dealing particularly with genetics, growth, behavior, developmental mechanics, sex determination, and hormonal interrelationships, and also for pure mammalian functional physiology and the physical chemistry of non-living systems. Preference will be given to analyses of fundamental physiological phenomena whether the material is vertebrate or invertebrate, plant or animal. Since the journal is restricted, it is not possible to publish more than a limited number of papers which must be short and concise.

It is recognized that prompt publication is essential, and the aim will be to issue papers within three months of acceptance.

Manuscripts and drawings should be sent to the Managing Editor, Dr. Arthur K. Parpart, P.O. Box 704, Princeton University, Princeton, New Jersey.

The paper must be accompanied by an author's abstract not to exceed 225 words in length, which will be published in Biological Abstracts. Nothing can be done with the manuscript until the abstract is received.

Manuscripts should be typewritten in double spacing on one side of paper 8½ × 11 inches, and should be packed flat — not rolled or folded. The original, not carbon, copy should be sent. The original drawings, not photographs of drawings, should accompany the manuscript. Authors should indicate on the manuscript the approximate position of text figures.

Manuscripts and drawings should be submitted in complete and finished form with the author's complete address. All drawings should be marked with the author's name. The Wistar Institute reserves the privilege of returning to the author for revision approved manuscript and illustrations which are not in proper finished form for the printer. **When the amount of tabular and illustrative material is judged to be excessive, or unusually expensive, authors may be requested to pay the excess cost.**

The tables, quotations (extracts of over five lines), and all other subsidiary matter usually set in type smaller than the text, should be typewritten on separate sheets and placed with the text in correct sequence. Footnotes should not be in with the text (reference numbers only), but typewritten continuously on separate sheets, and numbered consecutively. Explanations of figures should be treated in the same manner, and, like footnotes, should be put at the end of the text copy. **A condensed title for running page headlines, not to exceed thirty-five letters and spaces, should be given.**

Figures should be drawn for reproduction as line or halftone engravings, unless the author is prepared to defray the additional cost of a more expensive form of illustration. All colored plates are printed separately and cost extra. In grouping the drawings it should be borne in mind that, after the reduction has been made, text figures should fit one column width (2⅝ inches) or two column widths (5½ inches). Single plates may be 5 × 7½ inches, or less, and double plates (folded in the middle), 11½ × 7½ inches. Avoid placing figures across the fold, if possible.

Figures should be numbered from 1 up, beginning with the text figures and continuing through the plates. **The reduction desired should be clearly indicated on the margin of the drawing.**

All drawings intended for photographic reproduction either as line engravings (black-ink pen lines and dots) or halftone plates (wash and brush work) should be made on white or blue-white paper or bristol board — not on cream-white or yellow-tone. Photographs intended for halftone reproduction should be securely mounted with colorless paste — never with glue, which discolors the photograph.

Galley proofs and engraver's proofs of figures are sent to the author. All corrections should be clearly marked thereon.

Reprints may be obtained according to rates shown on a reprint order blank which will be sent with the proof to the author.

JOURNAL OF
CELLULAR AND COMPARATIVE
PHYSIOLOGY

VOL. 55 APRIL 1960 No. 2

CONTENTS

A. L. TAPPEL. Cytochromes of Muscles of Marine Invertebrates 111

PHILIP C. LARIS, GORDON NOVINGER AND JOHN CALAPRICE. Phloretin, Phloridzin and Glucose Inhibition of a Phosphatase on the Stromata of Rabbit Erythrocytes 127

TOSHIO NARAHASHI AND TERUO YAMASAKI. Behaviors of Membrane Potential in the Cockroach Giant Axons Poisoned by DDT 131

RICHARD T. JACKSON. The Olfactory Pigment 143

F. HERMANN RUDENBERG AND JULIAN M. TOBIAS. The Effect of Ribonuclease on the Dialysis of Calcium from Homogenates of Lobster Nerve and the Binding of Sodium in the Homogenates 149

S. SOLOMON AND JULIAN M. TOBIAS. Thixotropy of Axoplasm and Effect of Activity on Light Emerging from an Internally Lighted Giant Axon 159

JOSEPH V. PEARINCOTT. Changes in the Lipid Content During Growth and Metamorphosis of the House Fly, *Musca domestica* Linnaeus 167

F. R. HUNTER. The Effect of Tannic Acid on the Permeability of Erythrocytes to Non-electrolytes 175

GEORGE CAMOUGIS. Visual Responses in Crayfish. I. Recording Shock Responses to Light with Remote Electrodes 189

JOSEPH K. HICHAR. Spontaneous Electrical Activity in the Crayfish Central Nervous System 195

CAROLYN WELLS. The Response of *Tetrahymena pyriformis* to Ionizing Radiation: Strain Specific Radiosensitivities 207

PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America